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Genetic and Dietary Enhancement of n-3 Fatty Acids in Sheep Meat

By

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Declaration of Originality

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Abstract

Experiments were carried out to determine the effect of supplementing purebred and crossbred weaner lambs with *Spirulina* on fatty acid composition and the expression of fat metabolism and lipogenesis related genes in the muscle, adipose, liver, heart and kidney organs. The transcription levels of *Aralkylamine N-acetyltransferase (AANAT), Adrenergic beta-3 receptor (ADRB3), B-cell translocation gene 2 (BTG2)* and *Fatty acid synthase (FASN)* genes provided consistent evidence that 10% dietary supplementation with *Spirulina* increased prime lamb growth and meat quality through heavier liveweights, longer body lengths (BL) and enhanced polyunsaturated fatty acid (PUFA) compositions under pasture-fed management. Under simulated drought conditions when sheep lose weight and body condition, supplementation with *Spirulina* compensated for weight loss and sequestrated for sheep production losses. The transcription levels of *AANAT*, *ADRB3*, *BTG2* and *FASN* genes under simulated drought conditions assisted in shedding light on the underlying biological mechanisms at the molecular level; significant increases in n-3 and n-6 fatty acids were observed in lambs supplemented with 10% *Spirulina* indicating a positive impact on meat quality performance. A single nucleotide polymorphism (SNP) study was carried out to determine the effect of *ADRB3* gene mutations on the growth performance of purebred and crossbred sheep. SNP analyses revealed that Black Suffolk breed was less mutated compared to other breeds resulting in higher liveweight and an improved PUFA profile. Taken together, these results suggest that in crossbred sheep production, supplementation with *Spirulina*, a linoleic acid (LA) and α-linolenic acid (ALA) rich diet induced SFA depression and an increase in PUFA.

Thesis Publications

Peer-reviewed Journal Papers

1. Kashani A, Holman BWB, Nichols PD, Malau-Aduli AEO 2015. Effect of dietary supplementation with Spirulina on the expression of AANAT, ADRB3, BTG2 and FASN genes in the subcutaneous adipose and *Longissimus dorsi* muscle tissues of crossbred Australian sheep. *Journal of Animal Science and Technology*, 57:8 doi:10.1186/s40781-015-0047-3

2. Kashani A, Holman BWB, Nichols PD, Malau-Aduli AEO 2015. Effect of level of Spirulina supplementation on the fatty acid compositions of adipose, muscle, heart, kidney, and liver tissues in Australian dual-purpose lambs. *Annals of Animal Science*, 15 (4): 1-16 doi: 10.1515/aoas-2015-0037

3. Kashani A, Malau-Aduli AEO 2014. Real-time PCR and real-time RT-PCR applications in food labelling and gene expression studies. *International Journal of Genetics and Genomics* 2 (1): 6-12, doi: 10.11648/j.ijgg.20140201.12

4. Malau-Aduli AEO, Kashani A 2015. Molecular genetics and genomics-nutrition interactions in fatty acid composition of cyanobacterial microalga: Effect of dietary supplementation of purebred and crossbred Australian lambs with Spirulina (*Arthrospira platensis*) on the mRNA expression and transcriptional analysis of AANAT, ADRB3, BTG2 and FASN genes in the heart, kidney and liver. *Genes and Genomics*, 37 (7):633-644 doi:10.1007/s13258-015-0294-1

5. Malau-Aduli AEO, Holman BWB, Kashani A, Nichols PD 2015. Sire breed and sex effects on the fatty acid composition of heart, kidney, liver, adipose and muscle tissues of purebred and first-cross prime lambs *Animal Production Science*, 55 (11) doi: http://dx.doi.org/10.1071/AN14906

6. Holman BWB, Kashani A, Malau-Aduli AEO 2014. Effects of Spirulina (*Arthrospira platensis*) supplementation level and basal diet on liveweight, body conformation and growth traits in genetically divergent Australian dual-purpose lambs during simulated drought and typical pasture grazing. *Small Ruminant Research*, 120 (7): 6-14, doi http://dx.doi.org/10.1016/j.smallrumres.2014.04.014

7. Kashani A, Holman BWB, Malau-Aduli AEO 2015. Single Nucleotide Polymorphisms of the Ovine ADRB3 gene in crossbred Australian sheep supplemented with Spirulina (Arthrospira platensis) cyanobacterial microalgae. *New Zealand J. Agricultural Research* [Submitted]

Peer-reviewed /Edited Conference Papers

8. Malau-Aduli AEO, Kashani A 2015. *Arthrospira platensis*: A novel feed supplement influences gene expression in the hearth, kidney and liver of prime lambs. *Proceedings Association for the Advancement of Animal Breeding & Genetics*, 28-30 Sept 2015, Lorne, VIC, Australia, pp 1-4.

9. Malau-Aduli AEO, Flakemore AR, Holman BWB, Kashani A, Lane PA 2013. *Arthrospira platensis*: A novel feed supplement improves meat eating quality of Australian lamb. *Proceedings of the 11th World Conference on Animal Production*, 15-20th October 2013, Beijing International Convention Centre, Beijing, China, pp.121-122

10. Holman BWB, Kashani A, Malau-Aduli AEO 2013. Relationships between wool quality, body conformation and liveweight measurements in genetically divergent sheep supplemented with Spirulina. *Proceedings of the 11th World Conference on Animal Production*, 15-20th October 2013, Beijing International Convention Centre, Beijing, China.

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List of Abbreviations

The following abbreviations are used in this thesis:

AA, arachidonic acid

AANAT, Aralkylamine N-acetyltransferase

ACOXI, acyl-coenzyme A oxidase

ADF, acid detergent fibre

ADRB3, Adrenergic beta-3 receptor

ALA, α-linolenic acid

ANOVA, 1-way analysis of variance

BCS, body condition score

BTG2, B-cell translocation gene 2

BW, body weight

CLA, conjugated linolenic acid

DGLA, Di-homo-gamma linolenic acid

DHA, docosahexaenoic acid

DM, dry matter

DPA, docosapentaenoic acid

EPA, eicosapentaenoic acid

ETA, eicosatetraenoic acid

FA, fatty acid(s)

FASN, fatty acid synthase

FO, fish oil

GC, gas chromatography

GC-MS, gas chromatography mass spectroscopy

GDMI, grass dry matter intake

GH, growth hormone

GLA, γ-linolenic acid

LA, linolenic acid

LC, long chain ($\geq C_{20}$)

mRNA, messenger ribonucleic acid

MUFA, monounsaturated fatty acid(s)

NDF, neutral detergent fibre

NEB, negative energy balance

PUFA, polyunsaturated fatty acid(s)

RT-PCR, real time quantitative polymerase chain reaction

SDA, stearidonic acid

SFA, saturated fatty acid(s)

UFA, unsaturated fatty acid(s)

n-3, omega 3

n-6, omega 6

MTTP, Microsomal triglyceride transfer protein

FABP4, Fatty acid binding protein 4

SCD, Stearoyl-CoA desaturase

MSTN, Myostatin

AANAT, Aralkylamine N-acetyltransferase

ADRB3, Adrenergic beta-3 receptor

BTG2, B-cell translocation gene 2

FASN, Fatty acid synthase

Chapter 1

General Introduction

Governor Philip brought 29 sheep into Australia on the first fleet over 200 years ago (Kauffman, 1938). He bought them from the Cape of Good Hope in South Africa. The colonists discovered that sheep farming conditions in Australia were different from England because the climate was hot and grasses around Sydney did not fit the sheep diets (Kauffman, 1938). There are now around 117 million sheep in Australia. (Newman *et al.*, 2002).

Escalating meat prices over the last few years and low wool prices have brought the Merino industry into a crisis which has resulted in an unexplained decline in Merino ewe numbers. As an alternative strategy, more flocks are being mated to meat sires (Leymaster, 1987). One of the important components of lamb production in Australia is crossbred ewes. Annually, over 5 million ewes are mated to terminal sire rams (Afolayan *et al.*, 2009). In 2001 and 2002, 59% of slaughtered lambs were of Merino breed which provided at least 80% of the prime lamb dam genotypes, and current statistics show that 99.9% of sheep meat produced in Australia is Merino-based (Huisman and Brown, 2009).

Crossbreeding is the systematic usage of breed resources to develop offspring of a particular type (Schoenian, 2009) by mating males and females of different breeds. Crossbreeding of sheep exploits the use of both additive and non-additive genetic effects (Newman *et al.*, 2002). The goal is to accomplish optimal degrees of function appropriate for outlined systems of sheep production and marketing (Leymaster, 1987). In this regard, accurate selection is one of the most important strategies for maximising production; thus, precision in the estimation of

genetic parameters is necessary for predicting genetic gain (Lôbo *et al.*, 2009). Effective crossbreeding program is defined as one that exploits breed differences and capitalises on the combined effects of individual and maternal heterosis and breed complementary. Breed differences are exploited by efficient crossbreeding systems, maternal heterosis and complementarity, and capitalizing on the effects of the individual (Freking *et al.*, 2000).The benefits of a dual-purpose sheep in terms of genetic response, are increased profitability due to the improvement in lamb weight, wool production and reproductive ability (Brash *et al.*, 1994; Crouse *et al.*, 1981; Freking and Leymaster, 2004). The rate of muscle and fat development in sheep can change with genotype, breed, management practices, age, and nutrition (Ponnampalam *et al.*, 2007). Knowledge of economically important genetic parameters are needed for development of effective genetic evaluation and improvement programs (Safari *et al.*, 2005). Large across-generation data sets for each population are required for the accurate estimation of genetic parameters, which are not always available (Safari *et al.*, 2005), but if there is consistency across populations, pooling estimates from several populations may provide more authentic parameter estimates than those found from a single population (Safari *et al.*, 2005).

Single Nucleotide Polymorphism discovery in cattle (Bovine Genome Sequencing and Analysis Consortium *et al.*, 2009; Van Tassell *et al.*, 2008) has not only facilitated paternity validation (Werner *et al.*, 2004; Heaton *et al.* 2002), but has also provided a cutting-edge technology for livestock traceability (Karniol *et al.* 2009) and characterization of linkage disequilibrium (Khatkar *et al.*, 2007). A large number of polymorphisms between different populations have been found through the completion of The Human Genome Project. Most of these variations are single nucleotide polymorphisms, of which more than 9 million have been reported in public databases (Kim and Misara, 2007). Single nucleotide polymorphisms

involve a single nucleotide change at one position of the genetic code and are therefore simpler forms of DNA variation than microsatellites (Allen *et al.*, 2010).

DNA markers that could be employed for genetic profiling comprise microsatellites, single nucleotide polymorphisms (SNPs), restriction fragment length polymorphisms (RFLP) and mitochondrial sequence polymorphisms (mtDNA) (Bowling, 2001).“Microsatellites are short sequences of 1-5 base pair motifs repeated in a head-to-tail arrangement from two to 40 times, the so-called short tandem repeats” (Mommens *et al.*, 1998). Microsatellite-based tests have many advantages such as distinguishing between a large number of alleles, high polymorphic information content, and ease of genotyping (Ozkan *et al.*, 2009). Thus, microsatellite markers have been the popular marker of choice for parentage assignment. However, microsatellites have a high mutation rate that makes them genetically unstable and liable to misclassification in animal parentage testing (Hara *et al.*, 2010). As alternatives, Single Nucleotide Polymorphic (SNP) markers have lower mutation rates (Kim and Misara, 2007), lesser genotyping errors (Weller *et al.*, 2010), more genetic stability (Donthu *et al.*, 2010), more amenability to high-throughput automated analysis (Lin *et al.*, 2010) and more robustness in laboratory handling and data interpretation than microsatellites (Allen *et al.*, 2010).

Broad scale DNA testing needs the development of fast, automated and small devices (Irizarry *et al.*, 2003). Gene-chips are biosensors that provide fast, selective, and sensitive detection of DNA hybridisation (Kim and Misara, 2007). A combination of gene-chips is called a DNA microarray that consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides used in molecular biology and known as probes (Irizarry *et al.*, 2003). This probe can be a short part of a gene or other DNA component that is used to hybridize a cDNA or cRNA sample (Kim and Misara, 2007). Probe-target hybridization can be quantified by the

detection of chemiluminescence-labeled or fluorophore-silver targets to find proportional abundance of nucleic acid sequences in the target (Kim and Misara, 2007). Since an array contains thousands of probes, a micro array can fulfill many genetic tests in parallel (Butler *et al.*, 2001). DNA microarrays are used to evaluate changes in expression levels to find single nucleotide polymorphisms (SNPs) (Elnifro *et al.*, 2000). The essential principle of microarray is hybridization between two DNA strands and complementary in nucleic acid sequences (Irizarry *et al.*, 2003) which identifies the SNP between alleles among or within populations (Leymaster, 1987). Microarrays use SNP detection for several applications, including forensic analysis, identifying drug candidates, genotyping, measuring predisposition to disease, evaluating somatic mutations in cancer or germline mutations in individuals and assessing loss of heterozygosity in genetic linkage analysis (Michael, 1999).

One of the most important goals of profitable livestock production is meeting consumer requirements and improving the quality of marketed carcass (Hopkins *et al.*, 2007). The Australian sheep industry has experienced significant changes over the last 20 years with a steady rise in price and production of lamb and mutton and a sustained reduction in the scale and value of the wool industry (Rowe, 2010). This increasing economic value of meat compared to wool makes breeding objectives for sheep enterprises more complex (Safari *et al.*, 2005). Distinctly defined breeding targets not only simplify decisions, but they are also necessary for developing breeding strategies that determine accurate selection criteria for the applicable traits that lead to commercial farm profitability (Byrne *et al.*, 2010). According to the marketing research undertaken by Meat and Livestock Australia, there are five key points that can be attributed to modern meat products that are linked to customer interest (Pethick *et al.*, 2010):

- Products should have a high organoleptic appeal such as tenderness, juiciness, and flavour to result in a satisfactory level of eating quality
- They must enhance general health conditions by having a good source of lean high quality protein and nutrition
- They must be produced within ethical production systems that meet food safety and traceability guidelines
- The production system relating to the supply chain and customer demands should be efficient (i.e., of good value).

A very important factor that determines the financial value of a carcass along the supply chain is saleable meat yield, that is, a use of the weight of muscle compared to the weight of the carcass (Gardner *et al.*, 2010). In Australia, lambs for meat production are primarily from first-cross Poll Dorset × Border Leicester × Merinos, second-cross Poll Dorset × Merinos/ Border Leicester × Merinos and directly Merinos (Ponnampalam *et al.*, 2008). Afolayan *et al.* (2009) stated that: "The major issues confronting red meat consumption are the need for simultaneous improvement of lean meat yield, eating quality and human nutritional value of the product". Improvement in this context refers to demand and willingness of the customers to pay for good quality meat that adds value to overall efficiency across the supply chain of increasing lean meat yield (Afolayan et al., 2009, Banks and Brown, 2009). The key productivity driver for meat supply chains is the amount of lean meat that can be boned out from a carcass. Consumers desire meat with less salvage fat, and this is most efficiently attained by producing relatively leaner slaughter animals on farms (Afolayan et al., 2009, Byrne et al., 2010, Daetwyler et al., 2010).

Therefore, the overarching objective of this thesis was to investigate the effect of supplementation with *Spirulina* on liveweight, body measurements, fatty acid (FA) compositions and relative gene expression of fat related genes in the muscle, adipose, heart, kidney and liver tissues of genetically divergent Australian purebred and crossbred prime lambs. The thesis is structured into the following chapters:

Chapter 1: General Introduction

Chapter 2: Literature Review: The literature review is an in-depth exploration of the utilisation of real-time PCR for gene expression studies and food labelling.

The successive chapters are investigative experimental studies that describe the effect of dietary supplementation with *Spirulina* on FA composition and gene expression in the various tissues of prime lambs under grazing and simulated drought conditions.

Chapter 3: The main objective of this chapter was to investigate the effect of *Spirulina* supplementation on the mRNA expression patterns of genes controlling lipid metabolism in the subcutaneous adipose tissue (SAT) and *Longissimus dorsi* (ld) muscle of Australian crossbred sheep.

Chapter 4: In this chapter, the main objective of the study was to assess the effect of dietary *Spirulina* supplementation on the messenger RNA expression profiles of genes controlling fatty acid metabolism in the heart, kidney, and liver of Australian purebred and crossbred prime lambs.

Chapter 5: The hypothesis tested in this study was that supplementation with *Spirulina* will compensate for the drought condition feed loss and assist in increasing live weight, growth, and body conformation.

Chapter 6: This study investigated the effects of *Spirulina* supplementation on the FA compositions of subcutaneous adipose, *Longissimus dorsi* muscle, kidney, heart, and liver tissues in purebred and crossbred Australian sheep.

Chapter 7: Investigated the effects of *Spirulina* on the expression of *Aralkylamine N-acetyltransferase* (AANAT), *B-cell translocation gene-2* (BTG2) and *Fatty Acid Synthase* (FASN) genes in the muscle, adipose, liver, kidney and heart tissues. The hypothesis tested was that supplementation with Spirulina will alter the expression of AANAT, BTG2 and FASN.

Chapter 8: This chapter is a general discussion and conclusion of the main thesis outcomes and areas warranting further studies.

Chapter 2

Literature Review

Spirulina

Introduction

Spirulina is a microalga that was first extracted by Turpin in 1827 from a freshwater stream. Its species have been found in a variety of environments, including soil, sand, marshes, brackish water, seawater, and freshwater. The adaptation to very different habitats and colonies is because the species of *Spirulina* have been sequestered, for instance, from tropical waters to the North Sea, thermal springs, salt pans, warm waters from power plants, fish ponds, etc. This cosmopolitan species can even live in environments that are very difficult, or even impossible, for other organisms live in (Ciferri, 1983). Some examples of these difficult environments include the lakes located in Kenya and Tanzania, including Nakuru, Elmenteita, Bogoria, Simbi, Reshitani, and Big Momella (Ciferri, 1983). *Spirulina* was first used in 1940 when these microalgae were found in a mast of floating microscopic algae on the surface of small lakes or ponds around Lake Chad. On that time it was eaten by local people who called it Dihd (or did) in the local language (Kanenbou) (Ciferri, 1983). As mentioned before, it had long history as a food in the alkaline lakes of Mexico and Africa where it was a source of food for their ancient human residents (Holman, 2013).

Increasing trends of global population, limited areas and regions of land that can be used for agricultural purposes, and negative effects of climate change on water resources and animal feed availability all create the need for animal products to supply food for the huge population, while also meeting the consumers' taste and developing markets in this section. This is especially true in developing countries that are in high demand, and therefore researchers

continue to seek a suitable source and supplement to meet these goals (Holman, 2013). What is more, even today starvation and malnutrition are still a prevalent problem in considerable parts of the world. Also, it seems that the efforts of various "green revolutions" were incapable of satisfying the most basic human need: adequate nutrition (Ciferri, 1983).

In this situation finding and introducing a suitable nutrient that can lead us to a sustainable production is critical (Holman, 2013). Since *Spirulina* was used as nutriment by some populations in and near Chad and, possibly, by the inhabitants of Mexico before the Spanish conquest, the investigations about the possibility of using its two popular species (i.e., *S. platensis* or *S. maxima*) as a food source for human or animal began (Ciferri, 1983).

Spirulina as a food source

As mentioned previously, the need for a suitable nutrient rich food source is greater now more than ever. Therefore, a question arises: Can *Spirulina* extrinsically (e.g., production technology, yield) and intrinsically (e.g., chemical composition, toxicity) satisfy this demand?

Production of microorganisms such as *Spirulina* has some advantages. Firstly, it cannot be regarded as a threat for current agriculture in terms of using land. Furthermore, it is not very dependent on weather conditions, while its production is more efficient in terms of time and land (Ciferri, 1983). Nowadays, it seems to be old fashioned to say that there is still a protein gap, but it is probably certain that protein ingestion deficiency is one of the reasons for suffering the harmful effects of malnutrition. Unfortunately, the damages of this protein deficiency to growth and mental health can be very severe and irreversible especially in very early life including before birth. The point is that typical yields (e.g., rice, cassava, wheat) in some parts of the world where malnourishment and starvation is prevalent are still rich in terms of energy but poor and insufficient in terms of protein. Therefore, there are still some parts in the globe

that people may take enough calories during the day, but due to the lack of protein supply the physical and mental health, especially in children, is still under a serious threat. For instance, in many parts of Asia, the production of energy-supplying sources such as rice and wheat has increased remarkably over the last 2 decades, while the production of protein-rich food sources such as legumes has fallen radically. In this situation, the need to produce a protein-rich source of food that can be edible directly or indirectly for human consumption and that complete and balance (but not compete with) traditional agriculture can be tangible more than ever. In this regard, *Spirulina* can be used to achieve these goals as it is efficiently produced, it is very adapted to harsh environments, it emits no carbon dioxide in its production, is easily harvested, is rich in protein (up to 70% of the dry weight) and vitamins and growth factors, and is more easily digestible by animals than yeasts and unicellular algae (Ciferri, 1983).

Although the production of *Spirulina* is thoroughly explained later in this section, it seems to be enough to say that this microorganism is efficiently produced because the natural alkaline lakes that are places in which two species of *Spirulina* (*S. platensis* and *S. maxima*) grow plentifully are usually found in arid areas of the tropics and subtropics where malnutrition is often endemic. Also, production of *Spirulina* emits less carbon dioxide to the air than unicellular algae such as Chlorella, Scenedesmlus, and Euglena due to its requirement of very alkaline pH of the growth medium that reserve carbon dioxide in water. The other benefit of the high alkaline growth medium is that this high alkalinity can reduce the growth of a majority of other microorganisms, including those pathogenic to humans and other animals. Another feature that can make *Spirulina* a promising product to supply protein for human and animals is that it can be easily harvested due to the spiral shape of the trichome and the presence of gas vacuoles that result in the formation of floating mats (Ciferri, 1983). These floating mats can be easily harvested by gravity filtration; therefore, the energy requirements associated with the

recovery of single-cell microorganisms will reduce considerably. *Spirulina* is one of the richest protein sources of plant origin, but it is also worth to consider that the quality of its protein is among the best among the plants. Furthermore, it is an efficient source of vitamins and growth factors. For instance, its linoleic acid content, which is a growth factor for humans, is the highest after milk and the oil of the evening primrose (*Oenothera biennis*). Cells that are enclosed by a thin trichome sheath and by a murein-containing cell wall are the features that make *Spirulina* a more easily digested protein by animals rather than the protein in yeasts and unicellular algae (Ciferri, 1983).

Spirulina is awell-adapted microalga that can be produced in a land-efficient way. *Spirulina* has been tested as supplemental feed in the diet of some animals. These tests showed that *Spirulina* had a considerable effect on the nutrition of many species. Since the 1960s, *Spirulina* has become commercially mass produced worldwide (Holman and Malau-Aduli, 2013). Therefore, these microalgae have been considered a valuable resource of food for livestock in agricultural fields. Some researchers found that *Spirulina* had a remarkable impact on the improvement of health and welfare of some animals. This remarkable improvement on farmed animals is due to its protein-rich composition which made these microalgae one of the precious commercial products in agriculture (Holman and Malau-Aduli, 2013). This product can help farmers to improve productivity as it can provide essential fatty acids, amino acids, minerals, and carotenoids (Holman *et al.*, 2014). Gamma-linolenic acid, which is effective at improving several health benefits can be provided to the livestock by using *Spirulina* (Holman, 2013). The effect of *Spirulina* as a supplement had been studied on different animals (e.g., chickens, pigs, rabbits, ruminants). The following is a summary of results for each animal. The effect of *Spirulina* in chicken feed depends on what type of feed it replaces. For example, it has been observed that replacing *Spirulina* by dehulled soybean meal decreases growth rates. However,

if *Spirulina* is used instead of groundnut cake and fishmeal, the growth rate would not be changed. The other factor that is important in evaluating the effect of *Spirulina* in chicken growth rates is the amount used. As is proved by several studies, 50-100 g/kg of feed ration can maintain the typical growth rate, but more than 200 g/kg would reduce the growth rate of chickens. What is important here is that *Spirulina* can be effective at improving the health of chickens, and it can also be cost-effective. Regarding to the health benefits of *Spirulina*, macrophage and overall mononuclear phagocyte system functionality was increased in chickens that consumed *Spirulina*; thus, disease resistance was higher in those chickens. This disease resistance was observed even in low levels of *Spirulina* such as 10 g/kg feed ration. It was also observed that when using *Spirulina* there is no need to use typical vitamin-mineral premixes. Furthermore, the quality of product can be improved by using *Spirulina*. For instance, eggs are produced with lower total cholesterol content with *Spirulina* provision because *Spirulina* is full of antioxidants and n-3 PUFA content. Furthermore, increasing the levels of *Spirulina* can result in a more strengthened yolk colour of the egg. It is because zeaxanthin, xanthophylls, and other carotenoids pigments, particularly beta-carotene which accumulate within yolks, are high in the content of *Spirulina*. The other impact of *Spirulina* in the quality of farmed chickens is that it can increase the yellowness and redness of muscle. It should be used in levels of 1% of the total ration during the week prior to slaughter to attain broiler muscle tissue and the pigmentation which can be the best for the customer's preference (Holman, 2013). Another animal that can use *Spirulina* as a food supplement is the pig. Trials on the effect of *Spirulina* on pigs show different results. In one experiment the *Spirulina*-fed group showed a 9% higher rate of growth compared to control pigs, while in another experiment the difference in growth was not significant. It seems that differing experimental methodologies was the main reason of such inconsistency. A benefit of feeding pigs with *Spirulina* is that it increases their fertility. According to Holman (2013), boars that had been fed with *Spirulina* showed greater

overall sperm quality compared to controls, with increased volume (11%), motility, and post-storage viability (5%).

The next animal that can be fed with *Spirulina* is rabbit. Experiments on commercially farmed rabbit meat have shown that using *Spirulina* in rabbit diets did not influence growth or carcass yields. Although *Spirulina*-fed rabbits have an increased total feed consumption compared to control, the results that showed *Spirulina* was ineffective on growth may question the capability of feed rations containing *Spirulina* over conventional diets. Rabbits that were fed both low- and high-fat diets had an improvement on crude protein digestibility when 1% of *Spirulina* to total dry matter was used in their diet compared to controls. Therefore, the best result would be achieved in rabbit diets if *Spirulina* was used in a basic diet rich of nutrients in order to supply enough energy to fuel optimal growth rates.

Similarly the impact on chicken, *Spirulina* heighten the quality of rabbit meat.

For example, *Spirulina* can be regarded as a fundamental cause for increasing GLA and n-6/n-3 PUFA ratios within rabbit muscle FA profile. In addition to having health benefits for humans, GLA benefit the colour of meat according to the customer's preference as it can improve rabbit meat's oxidative stability. *Spirulina*-fed rabbits were also healthier as they showed higher oxyhaemoglobin levels than control groups (Holman, 2013).

An experiment with *Spirulina* using dairy cows showed that it can increase productivity. According to Holman (2013), cows had 21% more milk when they had *Spirulina* in their diet. Moreover, *Spirulina* affected the quality of milk. Cows fed with *Spirulina* had between17.6% and 25.0% more fat, 9.7% more protein, and 11.7% more lactose compared to a control group. In addition, the amount of SFA decreased, while UFA increased in *Spirulina*-fed cows. This

result is probably due to microbial protein synthesis, avoidance of rumen degradation, and the nutrient-rich composition of *Spirulina*. Overall, it can be said that *Spirulina* can heighten the health factors of milk (Holman, 2013).

According to (Holman, 2013), lower and higher amounts of *Spirulina* in the diets of lamb could affect the amount and quality of wool. As wool fibres are primarily composed of proteins, having a protein-rich supplement such as *Spirulina* would increase the YIELD (YIELD refers to the fibrous content of wool and attracts high price premiums).

Sire breed, supplementation level, and sexual interactions could have a significant impact on the quality of wool. It seems that medium level of *Spirulina* supplementation resulted in better quality wool lambs than a low level of *Spirulina* supplementation. The breed of lamb may interfere with obtaining results about the quality of wool when feeding with *Spirulina.* For example, on a typical pasture-based basal diet, Black Suffolk sired lambs had higher wool than Dorset-sired lambs (Holman, 2013). In general, it can be said that feeding lambs with *Spirulina* can improve clean fleece yields, while other wool qualities such as fibre diameter, wool comfort factor, and spinning fineness are not affected (Holman, 2013). Also, using *Spirulina* leads to a faster growth of wool.

Birds such as flamingos were also investigated. The investigation on the consumed food in their stomach showed that in lakes containing *Spirulina*, during the periods in which the collections were made, the birds were feeding entirely on *Spirulina*. Furthermore, when *Spirulina* was abundant, the birds fed on the lakes and stayed in the area to breed. However, when the *Spirulina* decreased in Rift Valley lakes due to a change in the concentration of

chemicals in the water, the birds had to consume other cyanobacteria or benthic diatoms (Ciferri, 1983).

According to (Ciferri, 1983), the disappearance or the reduction of the *Spirulina* population causes the bird flocks to disperse to other bodies of water in eastern and southern Africa.

Morphology and taxonomy

Spirulina is a multicellular, filamentous cyanobacterium that under the microscope seems like blue-green filaments with cylindrical cells arranged in unbranched, helicoidal trichomes. Different genus of *Spirulina* can be distinguished by the helical shape of the trichome, but the helical parameters such as area length and helix dimensions can be different in various species. However, even within the same species, some differences have been observed in these parameters that may be induced by changing the environmental conditions such as growth temperature (Ciferri, 1983). One of the indicators that can be used in the classification and generic assignment of these organisms is the presence or absence of the septa. The smaller species have homogeneous cytoplasm without gas vacuoles or inclusions, and the septa is not clearly visible. *Spirulina platensis* and *Spirulina Maxima* are among two well-known species of *Spirulina*. The diameter of the helix is 35 to 50 µm in *S. platensis,* and it is 50 to 60 µm for *S. maxima* with a pitch of 60 µm and 80 µm, respectively. On the other hand, cell dimensions were greater in *S. platensis* than in *S. maxima* (diameter of 6 to 8 µm in the former and 4 to 6 µm in the latter). In contrast with smaller species, these species have a granular cytoplasm containing gas vacuoles and easily visible septa. According to electron microscopy of ultrathin sections of *S. platensis*, the cell wall was composed of four layers. The outer layer (L-IV) is composed of materials similar to the trichome axis and is considered analogous to that present in the cell wall of gram-negative bacteria. Layer III probably contains protein fibrils wound

helically around the trichomes. However, there is a peptidoglycan containing layer 2 (L-II) that bends towards the inside of the filament, giving rise, together with a putative fibrillar inner layer 1 (LI), to the septum separating the cells. The septum is like tinny floppy and is folded in part. This fold covers a portion of the septum surface, and its extent seems to be related to the pitch of the trichome. As the pitch gets larger, the folded area become smaller and vice versa. The fold coverage of the total septum area is different in various species. For example, in *S. platensis* the fold covers 5% of the total septum area, while it only covers 3% in *S. laxissima* (Ciferri, 1983).

The life cycle

Spirulina has a relatively simple life cycle in laboratory culture. The process starts with a break in a mature trichome into several pieces through the formation of specialized cells, called necridia, that undergo lysis and give rise to biconcave separation disks. Then a short chain of cells (i.e., two to four cells) is produced by the fragmentation of the trichome at the necridia. The hormogonia that move away from the parental filament can give rise to a new trichome. In the next stage, the cells in the hormogonium become rounded at the distal ends with little or no thickening of the walls by losing the attached portions of the necridial cells (Ciferri, 1983). During this process, the cytoplasm appears less granulated, and the cells assume a pale blue-green colour. The number of cells in hormogonia increases by cell fission while the cytoplasm becomes granulated, and the cells assume a brilliant blue-green color. By this process, the trichomes increase in length and assume the typical helicoidal shape. Random but rare spontaneous breakage of trichomes, together with the formation of necridia, assures growth and dispersal of the organism (Ciferri, 1983).

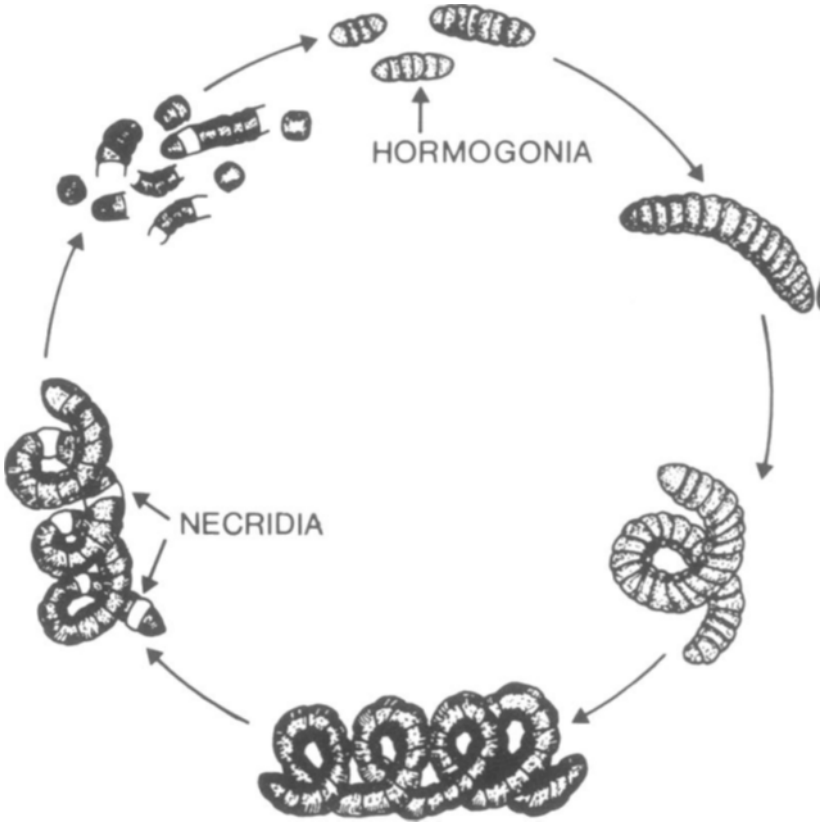

Figure 1.1 Life cycle of *Spirulina* (Ciferri, 1983)

Production of Spirulina

The only plant currently known to produce *Spirulina* is located in Lake Texcoco. Lake Texcocois is located 2,200m above sea level in a semitropical climate in the Valley of Mexico. Its average annual temperature is 18°C. The species of *Spirulina* that grows there naturally is *S. maxima*. The firm that runs a production plant to extract soda from the lake is now recovering, and commercializes the cyanobacterial biomass. The most external portion of a giant solar evaporator of spiral shape with a diameter of 3 km and a surface area of 900 ha is the place that *S. maxima* is harvested. Filtration is used to recover the biomass. In the next stage, it is homogenized and pasteurized and spray dried. Approximately 2 tons is the rate of its daily production with a yield of 28 tons of protein/ha per year. The long-term goal of producing and consuming *Spirulina* seems to be finding a suitable protein source for human consumption.

However, up until now it appears that *S. maxima* biomass is commercialized mostly as a feed for animals (Ciferri, 1983).

Another important benefit of *Spirulina* is that it can be grown within a nutrient-rich liquid medium; hence, its production is highly land-use efficient. For example, *Spirulina* can be the outcome of many traditional livestock feeds, such as wheat, corn, barley, and soybeans, in protein output per land unit. Moreover, desalinated wastewater or a growth medium enriched with animal faecal waste is suitable for *Spirulina* growth. In this case, faecal waste of pigs and cattle were used in an experiment, and the extracted *Spirulina* was safe enough to consume. Therefore, in this way waste can be used to produce a nutrient (Holman, 2013). The benefit of using wastewaters to grow *Spirulina* is that it provides an efficient way to couple protein production, recycling of nutrients removal of organic and inorganic pollutants, and disposal of wastes. According to some experiments, it is possible to grow *S. maxima* and *S. platensis* on city wastewaters, cow manure, or swine wastes. This is a very cost effective way to produce nutrients. Another interesting way of producing *Spirulina* is by using carbon dioxide-enriched air. However, there is not enough information about practical applications regarding this. Still, it seems fair to say that during the long years success in the recycling of industrial or urban wastewaters and also producing of protein biomass can be an attractive subject in the future (Ciferri, 1983).

Unfortunately, producing and purchasing *Spirulina* is expensive compared with many kinds of livestock feed. Therefore, it is not used in many animal production systems. Although *Spirulina*'s palatability, its dry powdery form, and its smell further limit its practicality, there are some current improvements on low-cost growth media and improved operational management that reduce the cost of producing and using *Spirulina* as it is an efficient nutrient

that can generate a high growth rate for livestock. Also research on the delivery methods and impact of *Spirulina* on product quality can give a better understanding of its capability in more efficient production (Holman, 2013).

In general, it seems fair to say that *Spirulina*, as a microalga that can be produced by using waste waters and arid areas, can be regarded as a sufficient food source that is rich in protein and other nutrients such as vitamins and growth factors. It is promising that in the future with enough research and information about the production and use of this valuable microalga, the current problems of malnutrition can be reduced in the near future, and global agriculture can move toward providing better food in terms of quality and quantity.

Fatty Acids: The fat content, fatty acid composition, and the effects of fatty acids on meat quality

Introduction

Red meat consumption is predicted to increase globally in the next couple of decades along with world population, income potential, and availability and accessibility to food to cope with human nutrient requirements as part of everyday diet (Kouba and Mourot, 2011, Mapiye et al., 2011, Woods and Fearon, 2009). Technological quality of fresh meat and the sensory quality of meat products are impressed by the composition of fatty acids (FAs), which also influences human health. Fat is an important constituent of meat for sensory perception of texture, juiciness, and flavour. In addition, fat in meat provides fatty acids that cannot be synthesised by humans (Wood et al., 2008, Wood et al., 2004). Although socio-cultural tastes of consumers evaluate meat quality, fat plays an essential role in meat's eating quality, as it determines meat

flavour, tenderness, and juiciness (Perez et al., 2010, Smet et al., 2004). Hence, fatty acid composition plays a significant role in the definition of meat quality (Santos-Silva *et al.*, 2002).

The fat in adipose and muscle tissue of sheep meat contains saturated (SFA), mono-unsaturated (MUFA), and polyunsaturated (PUFA) fatty acids, and the range of SFA, MUFA and PUFA in lamb and mutton are 40 to 55%, 35 to 45%, and 1.5 to 7%.

Improving customer perception of texture and taste increases by an increase of intramuscular fat mostly in loin meat (Perez *et al.*, 2010). On the other hand, fat is an unpopular part of meat for consumers in some countries and is believed to be unhealthy. However, fat and fatty acids, whether in muscle or adipose tissue, are central to the nutritional value of meat and contribute significantly to several aspects of meat quality (Wood et al., 2008, Kouba and Mourot, 2011). In animal products the proportion of polyunsaturated FAs (PUFAs) is seen by consumers as important for dietetic value of the meat (Perez *et al.*, 2010). Thus, although a reduction in total fat intake is recommended by scientific evidence and nutritional guidelines (Mapiye *et al.*, 2011), especially of saturated fatty acids (SFA) and *trans* fatty acids, nutritionists recommend consumers to increase intake of polyunsaturated fatty acids (PUFA) (Mapiye et al., 2011, Sasaki et al., 2006).

n-3 polyunsaturated fatty acids (n-3 PUFA) and n-6 polyunsaturated fatty acids (n-6 PUFA) are distinguished to have potential positive effects on human health and to be beneficial for human nutrition (Nguyen et al., 2010, Smet et al., 2004). The percentage of long-chain n-3 FAs in total FAs is called the n-3 index (O3I); an increase in the diet has been demonstrated to prevent and reduce the risk of cardiovascular disease, asthma, rheumatoid arthritis, cognitive decline, diabetes, osteoporosis, neurological dysfunction, and possible cancers (Nguyen et al.,

2010, Alfaia et al., 2009). Two important indexes for nutritional evaluation of fat are the ratio between polyunsaturated and saturated FA (P/S) and the ratio between n-3 and n-6 FA (Santos-Silva *et al.*, 2002). Diet, genetic, gender, age, hormones, and ambient temperature influence the proportion of nutrients and the composition of FAs in meat (Perez et al., 2010, Nguyen et al., 2010). The conversion of saturated fatty acids (SFA) into monounsaturated fatty acids (MUFA) is the fundamental process, and *Stearoyl-CoA desaturase* (SCD) is a key enzyme responsible for this conversion (Hoashi et al., 2008, Moibi and Christopherson, 2001)

As mentioned previously, several internal and external factors influence the quantity and quality of lipids in animal products. Internal factors such as genotype, age, gender, castration, and weight chiefly influence the quantity of lipid in meat products, and between these factors genetic component is very important (Kouba and Mourot, 2011, Sasaki et al., 2006). Genetic components are recognized to affect the biological characteristics of muscles such as intramuscular adipose tissue, fibre type, collagen, and prostate activities (Perez *et al.*, 2010). Results of many studies have recognized that diet plays a major role on modulating the fatty acid composition in tissues in both lambs and cattle (Cabiddu et al., 2006, Hoashi et al., 2008). As Perez *et al.* (2010) has indicated, feeding and selection are two major methods that have been utilized to control lipid content and the composition of fatty acids regarding the importance of carcass value and meat quality.

Regarding feeding, some tests have been suggested to alter the fat ratio in muscle by diet supplementation with linseed, fish oil, and pasture grass, but the results were inconsistent (Perez *et al.*, 2010). In other nutritional studies, diets have been manipulated to contain different fat lipids or oils, flaxseed (common FA in grain and oil-seeds), vegetable oils high in oleic acid, high levels of linoleic acid (18:2n-6), soybean oil high in linoleic acid, and palm oil high in palmitic and palmitoleic acids, namely, (16:0) and (16:1) (Nguyen et al., 2010, Sasaki

et al., 2006, Woods and Fearon, 2009). A desirable approach with an object of increasing 18-carbon fatty acids is the manipulation of the fatty acid composition of ruminant tissues (Moibi and Christopherson, 2001). According to Kouba and Mourot (2011), genetic makeup is an important component of the manipulation of fatty acids' quantity and quality. For example, in pigs, breeding for leaner carcasses has resulted in a reduction of total fat from 35 to 45% to less than 20%. In addition, selective breeding results in changes in fat distribution among different depots, which produces animals with lower subcutaneous fat without diminishing intramuscular fat (Kouba and Mourot, 2011, Moibi and Christopherson, 2001).

Diet

Defining the diet that best meets the needs of the human species has always been one of the most interesting challenges of modern biology (DeBusk, 2010). Hoashi (2008) states that in a study on cattle, Zembayashi presented that adipose tissue of Japanese black cattle contains a higher proportion of MUFA than that of Japanese brown cattle, Holstein, or Charolais. As a result, fatty acid composition may be controlled by genetic factors such as fatty acid metabolism and lipid synthesis related genes. As already mentioned, fatty acid composition in meat producing animals has been considered as a significant factor influencing human health. Fatty acid composition is influenced by genetic makeup, environmental factors, and management; nevertheless, feeding and genetics are considered to be the main source of alteration (Barzehkar et al., 2009 , Smet et al., 2004). In this paper, an attempt was made to review feeding and genetic effects on muscle fatty acid composition.

Genetic variability

Differences between species, breeds or lines, crossing of breeds, and between animals within breeds creates genetic variability. Heritability and genetic correlation are estimated as the latter origin of variation. (Boucher et al., 2006, Nguyen et al., 2010, Smet et al., 2004).

Fatty acid composition of muscle and adipose in meat animals

The total fatty acid content and fatty acid composition of subcutaneous adipose tissue and *longissimus* muscle purchased at retail from loin chops or steaks of pigs, sheep, and cattle are shown in Table 2.1 (Wood *et al.*, 2008). The data demonstrate that muscle has much lower fatty acid content than adipose tissue; however, the fatty acid composition of the two tissues is broadly similar. Furthermore, it is interesting that the proportion of the major polyunsaturated fatty acid (PUFA) and linoleic acid in pigs is much higher than in sheep and cattle (Wood *et al.*, 2008). Grains and oilseed fatty acids are degraded into monounsaturated and saturated fatty acid by rumen of ruminants by microbial biohydrogenation, and just about 10% of dietary 18:2*n* -6 is usable for incorporation into tissue lipids (Wood et al., 2008, Kouba and Mourot, 2011). Significant proportions of long-chain (C20-22) PUFAs are in muscles that are made from 18:2n-6 and 18:3n-3 by the activity of elongase and Δ5 and Δ6 desaturase enzymes (Wood *et al.*, 2008). *Lipid digestion in ruminants and non-ruminants*

Transference of fatty acids from the diet into the animal product is affected by the type of lipid digestion by animals. In non-ruminants, the main site of digestion of dietary lipids is the small intestine. Mainly, triacylglycerols are broken from pancreatic lipase down to 2-monoacylglycerols, the formation of micelles acids absorption, lipid uptake is mediated by lipoprotein, and free fatty acids are spread throughout the body.

Table 2.1 Fatty acid composition (g/100 g fatty acids) and content (g/100 g total fatty acids in subcutaneous adipose tissue and muscle) of loin steaks/chops in pigs, sheep, and cattle adopted from (Wood *et al.*, 2008)

	Adipose tissue			Muscle		
	Pigs	Sheep	Cattle	Pigs	Sheep	Cattle
14:0	1.6[a]	4.1[b]	3.7[b]	1.3[a]	3.3[c]	2.7[b]
16:0	23.9[b]	21.9[a]	26.1[c]	23.2[b]	22.2[a]	25.0[c]
16:1cis	2.4[a]	2.4[a]	6.2[b]	2.7[b]	2.2[a]	4.5[c]
18:0	12.8[a]	22.6[b]	12.2[a]	12.2[a]	18.1[c]	13.4[b]
18:1cis 9	35.8[b]	28.7[a]	35.3[b]	32.8[a]	32.5[a]	36.1[b]
18:2n- 6	14.3[b]	1.3[a]	1.1[a]	14.2[b]	2.7[a]	2.4[a]
18:3n- 3	1.4[c]	1.0[b]	0.5[a]	0.95[b]	1.37[c]	0.70[a]
20:4n- 6	0.2	ND	ND	2.21[b]	0.64[a]	0.63[a]
20:5n- 3	ND	ND	ND	0.31[b]	0.45[c]	0.28[a]
n _ 6:n- 3	7.6	1.4	2.3	7.2	1.3	2.1
P:S	0.61	0.09	0.05	0.58	0.15	0.11
Total	65.3	70.6	70.0	2.2	4.9	3.8

[a,b,c] Means with different superscripts are significantly different ($P<0.05$). ND, not determined

Unlike in non-ruminants, fatty acids are unaltered before incorporation into the tissue lipids. Thus, in non-ruminants such as pigs and poultry, dietary lipid sources have a direct effect on the fatty acid composition, which is predictable (Woods and Fearon, 2009, Kouba and Mourot, 2011). In contrast, the composition of fatty acids left rumen for absorption in the small intestine are affected by the rumen microorganisms in the ruminant digestive system (Woods and Fearon, 2009).

Fatty acid sources in animal feedstuff

Essential fatty acids and fat-soluble vitamins are supplied by the fat in the animal's diet. Fat in animal's diet is important not only for this but also because of its energy rich contribution, which is approximately twice that of carbohydrates (Woods and Fearon, 2009, Mapiye et al., 2011, Smet et al., 2004). According to Woods and Fearon (2009), there are a number of deciding factors for the choice between fat or oil and its form in the provided feed for animals.

These include (1) raw materials cost and availability, both locally and international; (2) the impact of oil or fat form and its fatty acid composition on feed digestibility; (3) the view of consumers and retailers regarding the introduction of genetically modified materials into the food chain; and (4) animal feed regulations about allowed supplements (Afolayan et al., 2009, Kouba and Mourot, 2011).

Fatty acid composition in ruminants

Unlike non-ruminants, the fatty acid composition in ruminants is much less dependent on diet. The majority of the dietary unsaturated fatty acids are hydrogenated by the microorganisms within the rumen, and most dietary fatty acids are absorbed as saturated fatty acids. Nevertheless it is known that diet also influences the fatty acid composition of ruminants (Mannen, 2011, Perkins et al., 2006, Wong and Medrano, 2005). For example, in some studies on Japanese Black cattle have demonstrated that adipose tissue from Japanese Black cattle contains a higher proportion of MUFA than that of Charolais, Holstein, or Japanese Brown cattle. Also, it has been demonstrated that sire groups in Japanese Black cattle have a significant effect on fatty acid composition (Anderle et al., 2003, Asyali et al., 2006, Mannen, 2011). In addition to various environmental effects, these outcomes suggest that genetic factors such as lipid synthesis and fatty acid metabolism-related genes can control fatty acid composition (Bagnall and Kotze, 2010, Barendse et al., 2009, Mannen, 2011).

Lipid sources for ruminants

Woods and Fearon (2009) state that "the contribution of the basal diet to the fatty acid composition of the animal production can be difficult to quantify."

Table 2.2 Fatty acid profile of forages (g/100t g total FA) unless otherwise stated adopted from (Woods and Fearon, 2009)

Forage	Oil content (g/kg DM)	16:0	18:0	18:1*c*	18:2	18:3	Reference
Grass	29	20.8	3.29	5.74	14.0	49.2	French *et al.* (2000)
Grass silage	28	24.0	2.90	6.32	14.5	46.2	French *et al.* (2000)
Red clover (2003) (Autumn)	23	31.1	4.81	8.00	21.4	33.6	Loor *et al.*
Red Clover (2003) (Spring)	27	24.2	4.35	5.21	19.1	45.9	Loor *et al.*
White clover		3.99	0.50	0.34	3.41	4.21	Collins *et al.* (2002)

[a] Fatty acid contents for white clover are expressed as mg/g dry weight

The enzyme responsible for conversion of saturated fatty acids into MUFA in mammalian adipocytes is Stearoyl-CoA desaturase (SCD). The earlier action of SCD on substrates such as palmitic acid or stearic acid effects on the composition of fatty acids stored in fat depots (DeBusk, 2010, Mannen, 2011). Although, nutrition has clearly demonstrated to contribute to the fatty acid profile of intramuscular fat and subcutaneous fat, the genetic factors mostly determine the fatty acid profile (Bagnall and Kotze, 2010, Derveaux et al., 2010, Mannen, 2011).

Microsomal triglyceride transfer protein

The microsomal triglyceride transfer protein is a heterodimeric protein that plays a crucial role in the assembly of nascent lipoproteins in the liver and intestine (Vandesompele *et al.*, 2002). Its role is presumed to be the transport of triglycerides from the endoplasmic reticulum (ER) membrane to intra-ER nascent lipid droplets. In addition, it is suggested that MTTP participates in the expansion of nascent lipoproteins by direct transfer of triacylglycerols (Hocquette et al., 2009, JinQuan and WenGuang, 2009, Vandesompele et al., 2002).

Table 2.3 Significant genes involved in fat metabolism and synthesis

Gene	Symbol	Species	Effect	Reference
microsomal triglyceride transfer protein	*MTTP*	Pig	Fatty acid composition of porcine fat	(Estelle *et al.*, 2009)
Fatty acid binding protein 4	*FABP4*	Bovine, Holstein steers	Intramuscular fat of mammals, Fatty acid composition	(Barendse *et al.*, 2009, Mannen, 2011)
stearoyl-CoA desaturase	*SCD*	Cattle	Fatty acid composition of intramuscular fat	(Mannen, 2011)
Myostatin	*MSTN*	sheep	Regulation of adiposity and in controlling the structure and function of tendons	(Hickford *et al.*, 2010)
Aralkylamine N-acetyltransferas	*AANAT*	Sheep and Rat	Sleep regulation, reducing oxidative damage, protection against lipid peroxidation, controlling weight gain, and producing higher O3I levels	(Perez *et al.*, 2010) and (Coon *et al.*, 1999)
Adrenergic beta-3 receptor	*ADRB3*	Bovine and sheep	Birth weight, growth rate, carcass composition, cold survival, wool mean staple strength, and wool yield	(Forrest *et al.*, 2007a)
B-cell translocation gene 2	*BTG2*	sheep	Increasing n-3	(Reiter *et al.*, 2014) (Mo *et al.*, 2011)
Fatty acid synthase	*FASN*	human	Biosynthesis of long chain fatty acids, regulation of obesity	(Strosberg, 1997) (Wu *et al.*, 2011b, Berndt *et al.*, 2007)

By the action of lipoprotein lipase, free fatty acids are released from lipoproteins to the blood and are taken up by adipose tissues through specific fatty acid transporters (Vandesompele *et al.*, 2002).

Fatty acid binding protein 4

For many years, consumers have regarded tenderness as the most important property of meat quality. Traditional selective breeding is a difficult task to perform to increase meat tenderness because meat quality is controlled by polygenes (Hickford et al., 2010, Q.L. Xu et al., 2011). However, marker-assisted selection has helped for selection of meat quality traits. As Q.L. Xu *et al.* (2011) points out, "the intramuscular fat content (IMF) or marbling improves meat tenderness by reducing bulk density and decreasing the strength of the connective tissue, and has beneficial effects on the taste quality and juiciness". Thus, the genes involved in fatty acid metabolism are usually seen as potential candidate genes for meat quality.

In general, fatty acid binding proteins play a significant role in the regulation of lipid and glucose homeostasis, especially the fatty acid binding protein 4 (Q.L. Xu *et al.*, 2011). Fatty acid binding protein 4 initially identified as a candidate gene for obesity as it was located within a quantitative trait loci (QTL) region in mice and is a candidate gene that affects fatness traits of mammals (Barendse *et al.*, 2009). Later FABP4 had been shown to be a gene that affects intramuscular fat (IMF) in pigs.

Stearoyl-CoA desaturase

Stearoyl-CoA desaturase (*SCD*) plays a fundamental role in lipid metabolism and maintenance of membrane fluidity, based on the physical importance of the ratio between saturated and mono unsaturated fatty acids in humans and livestock (García-Fernández et al., 2009, Rockman and Kruglyak, 2006). Changes in the coding and/or regulatory sequences of the *SCD* in

ruminants can generate alterations in the enzymatic activity and can cause variations in the fatty acid content in milk and meat (García-Fernández et al., 2009, Moody, 2001). *Stearoyl-CoA desaturase* inserts a *cis* double bond between carbon 9 and 10 in the spectrum of saturated fatty acids preferring 16:0 and 18:0 (García-Fernández *et al.*, 2009). In lactating animals the *SCD* transcript is highly expressed in adipose tissue, the mammary gland, and the liver, and *SCD* is responsible for the production of about 80% of the most common form of conjugated linoleic acid (i.e., CLA, *cis*9, *trans*-11 18:2 isomer) in the mammary gland of ruminants (García-Fernández et al., 2009, Q.L. Xu et al., 2011).

Myostatin

Myostatin (known as the growth and differentiation factor 8 GDF8) is a regulator of myogenesis and a member of the transforming growth factor-beta family which covers a large number of growth and differentiation factors that play significant roles in regulating embryonic development and maintaining tissues homeostasis in adult animals (Hickford *et al.*, 2010).

Aralkylamine N-acetyltransferas

Aralkylamine N-acetyltransferas (AANAT) is responsible for encoding a supplementary protein. Its function is to interfere in melatonin synthesis and to control the night/day rhythm. Melatonin itself influences activity and sleep. Furthermore, melatonin stimulates the production of brown fat, which is a kind of fat that burns calories instead of storing them; therefore, it leads to weight loss. Melatonin also produces higher O3I levels as it protect PUFA from oxidative degradation in muscle tissue (Hu *et al.*, 2010). *Aralkylamine N-acetyltransferas* leads to the production of melatonin from serotonin, and greater amounts of melatonin within skeletal muscles further protect against oxidative degradation. Better understanding about the function of this gene that influences metabolic factors resulting long-chain n-3 fatty acid quantities in meat can lead to a better result in the healthiness of meat (Perez *et al.*, 2010).

Adrenergic beta-3 receptor (ADRB3)

Adrenergic beta-3 receptors are G-protein coupled receptors that usually can be found on the surface of adipocytes. They seem to play an important role in the regulation of energy balance. The body temperature can be regulated due to receptor stimulation which would result in non-shivering thermogenesis. Moreover, this receptor's stimulation can result in lipolysis in order to expend excess energy. As this gene is responsible for thermoregulation, its variations can lead to cold-related mortality (Forrest *et al.*, 2007a). In rodents, it is the main mediator of the lipolytic and thermogenetic effects of high catecholamine concentrations in brown and white adipose tissues (Hu et al., 2010, Kamaid and Giráldez, 2008). There are some studies that show a relation between the expression of *ADRB3* and obesity in humans and other mammals (Perez *et al.*, 2010).

B-cell translocation gene 2

B-cell translocation gene 2 *(BTG2)* is responsible for the regulation of cell cycle progression in a variety of cell types (Reiter *et al.*, 2014). This gene is known to be involved in cell growth, differentiation, and survival (Mo *et al.*, 2011). This gene is also known as PC3 and TIS21, which was primarily extracted as a basic gene induced by nerve growth factor (Mo *et al.*, 2011). It was also introduced as a tumour suppressor gene in humans and mice (Mo *et al.*, 2011). The protein that is encoded by this gene acts as a transcription co-regulator that can enhance or inhibit the activity of transcription factors; thus it is responsible for cell cycle progression control and pro-neural gene expression (Reiter *et al.*, 2014). Furthermore, several studies showed that *BTG2* has a possible impact on weight loss, intramuscular fat deposition, and muscle fibre size (Reiter *et al.*, 2014).

Fatty acid synthase

Fatty acid synthase *(FASN)* is responsible for encoding a multifunctional enzyme which is the catalyser in the synthesis of fatty acid (Strosberg, 1997). The enzyme that is encoded by this gene is known as a basic enzyme in *de novo* lipogenesis in mammals. The key role of this enzyme is being the catalyser in the synthesis of palmitate from acetyl-CoA and malonyl-CoA into a long-chain saturated fatty acid (LCSFA) in the presence of NADPH (Strosberg, 1997, Wu et al., 2011b). Also, the *FASN* gene plays a role in the development of obesity by contributing to the regulation of body weight (Strosberg,1997; Wu, 2012). The impact of FASN on obesity may occur through its role in regulation of feeding behaviour and energy homeostasis (Berndt *et al.*, 2007).

Gene Expression

One of the most popular domesticated animals in the world is sheep, and its meat, wool, milk, and fur skin are widely consumed. The meat, textile, and milk industries have gone through a revolutionary change, and these days significant considerations in selective breeding plans are multiple birth, rapid growth, high quality fibre, and good conformation (JinQuan and WenGuang, 2009). As Graziano *et al.* (2011) indicated, in animal breeding, it is relevant to identify the genetic and molecular basis of economically important traits, for this reason it is very helpful the identification of the genetic polymorphisms involved in different level of expression of a trait.

Genomics supplies scientists with methods to quickly study genes and their products. Using new biotechnological approaches, organisms' genes can be defined and their functions understood (Barendse et al., 2009, Hocquette et al., 2009). The genetic basis of complex and quantitative traits resists generalization even after more than a century of rediscovery of

Mendel. Basic questions including the number of loci that underlie variation in heritable phenotypes, their molecular nature and mechanisms of action and interaction, the distribution of their effect sizes, and their dependence on environmental variables remain unanswered (Rockman and Kruglyak, 2006). These questions are a major interest of medical and agricultural genetics issues as well as in basic evolutionary biology. Now genetic mapping of genome-wide gene expression has begun to provide the required empirical data to address these questions.

Since the first empirical linkage study of global transcript levels was published in 2002, many general principles have been found and constitute solid ground on which further study can be constructed (Rockman and Kruglyak, 2006). The emerging ability to sequence entire genomes has motivated researchers to not only to produce DNA sequences, but to also determine the function of genes on a genome-wide level; assumed that genes having related functions are probably to be regulated together (Moody, 2001, Anderle et al., 2003). Gene expression analysis has become significant in many fields of biological research. Realizing patterns of expressed genes is anticipated to furnish insight into complex regulatory networks and will most likely help to identify genes implicated in disease or relevant to new biological methods (Estelle et al., 2009, Vandesompele et al., 2002).

In other words, the blueprint of the structure and function of living organisms are genes that are the origin of encoded proteins that fulfil the work of the cell. Environmental factors and nutrient interactions with genes can alter functional outcomes. Thus, it is essential to understand the details of gene-nutrient interactions and how changes in a gene, or in the amount or form of a nutrient, determine functional outcomes (DeBusk, 2010, Asyali et al., 2006).

One of the significant factors that contribute to the meat quality of cattle and sheep is the deposition of intramuscular fat or marbling. This determines juiciness, flavour, and tenderness of cattle and sheep meat. The genetic background as well as their age and nutrition largely influence the accumulation of intramuscular fat (Asyali et al., 2006, Bagnall and Kotze, 2010, Wang et al., 2009). According to Wang et al (2009), the potential for cellular development of adipocytes is believed to be fixed relatively early in life and to change thereafter in either the size or number of cells that occur in proportion to the initial cell number and lipogenic proteins. Today the advent of genomics has given scientists the opportunity to examine the interaction between genes at the genome level and hence to realize the interactions between the various systems of a cell on a large-scale basis. This includes the interrelationship of a cell's DNA, RNA, synthesised protein and metabolites. Therefore, genomics helps to discover how these interactions are regulated (Barendse et al., 2009, DeBusk, 2010, Hocquette et al., 2009).

Genomics has shifted to the study of gene expression and function after the sequencing of a great number of genomes. Functional genomics permits the finding of genes that are turned on or off at any given time and in any nutritional or physiological situation (Derveaux et al., 2010, Estelle et al., 2009, Hocquette et al., 2009).

Techniques for gene expression studies

For many years scientists have looked for rate-limiting enzymes, a specific biological pathway, or key genes that have high impacts on physiological traits. Surely, molecular biology before genomics attempted to investigate the expression of single genes in isolation from the larger context of other genes (Derveaux et al., 2010, Estelle et al., 2009, Hocquette et al., 2009). This is denoted as the candidate gene approach. Today, different methods are used to discover and

quantify the expression level of individual genes, such as real-time PCR, northern-blot, or subtractive hybridization (Hocquette *et al.*, 2009).

Jin Quan and Wen Guang (2009) state that "Genetic structure is referred to as non-random distribution of gene or genotype in space and time, including genetic variation within population, genetic differentiation between populations, and other aspects".

Genomic selection in livestock

Throughout history, farmers have made small but significant genetic improvements to farm animals by selecting the best animals. The advent of genetics has resulted in higher rates of livestock improvement (Derveaux et al., 2010, Hocquette et al., 2009). In traditional genetics, farmers used information on phenotypes and pedigrees to predict breeding values. Then, in the 1990s genetic maps were developed that assisted in the discovery of QTL and even genes that control some production traits (García-Fernández et al., 2009, Hocquette et al., 2009). During this time commercial tools were originated, namely, single-markers and single-genes tests that have been quickly integrated into selection programmes (Graziano et al., 2011, Hocquette et al., 2009). However, the benefit made by marked-assisted selection (MAS) has been sometimes low depending on several factors:

- The accuracy of the existing estimated breeding
- The proportion of the genetic variance explained by the DNA markers
- The accuracy in estimating the effect of the QTLs
- The ability to reduce the generation interval by working at an earlier age (Hocquette *et al.*, 2009).

With the advent of genome sequencing, scientists identified SNPs in the genome of farm animals. According to recent databases and studies, now over 15,000 SNP markers covering all regions of all autosomes in more than 1,500 cattle have been analysed (Hocquette *et al.*, 2009).

Microarray Technology

Biologists use microarray techniques, through the application of genetic and molecular biology, to study global gene expression in cells and tissues in order to find key players in metabolic pathways and to assign the probable function of genes (Anderle et al., 2003, Pascual-Montano, 2011). Because of the recent advances in DNA microarray technology, today it is feasible to obtain gene expression profiles of tissue samples at relatively low costs. Microarray techniques are used by scientists around the world to characterize complex biological circumstances and diseases (Asyali *et al.*, 2006). Physicians and scientists use microarray techniques in genome-wide gene expression and gene mutation analysis to understand pathophysiological mechanisms and also in diagnoses and prognoses and when selecting treatment plans (Asyali et al., 2006, Graziano et al., 2011). In spite of the inaccessibility of this technology to animal scientists in the past, the resources required to produce livestock microarrays are being generated (Moody, 2001).

Real-time PCR for mRNA quantitation

Today reverse transcription quantitative PCR (RT-PCR) is regarded as the gold standard for gene expression in terms of accuracy, sensitivity, and fast results. Because of this, it distinguishes itself from other methods available for quantifying messenger RNA (mRNA) expression levels (Derveaux et al., 2010, Vandesompele et al., 2002, Wong and Medrano, 2005). As a result, real-time PCR has become one of the most widely used methods of gene quantization due to its advantages: it has a large dynamic range, it can be highly sequence-

specific, it is amenable to increasing sample, and it has little to no post-amplification processing (Anderle et al., 2003, Wong and Medrano, 2005). This technique provides reliable results depending on the use of an appropriate normalization method while analysing data (Bagnall and Kotze, 2010). The field of measuring gene expressions has been dramatically changed by the advent of real-time PCR and real-time PCR reverse transcription. In real-time PCR, data is collected throughout the PCR process as it takes place. Therefore, both amplification and detection are combined into a single step (Mannen, 2011).

To quantify gene expression, there are many benefits of using real-time PCR over other methods:

- Real-time PCR produces quantitative data with an accurate dynamic range of 7 to 8 log orders of magnitude, and it does not need post-amplification manipulation.
- In comparison to RNase protection assays, real-time PCR assays are 10,000 to 100,000-fold more sensitive.
- Real-time PCR compared to dot blot hybridization is 1000-fold more sensitive, and it can even find a single copy of a specific transcript
- Real-time PCR assays can reliably find gene expression differences as small as 23% between samples and have lower coefficients of variation (Wong and Medrano, 2005).

RT-PCR compared to conventional quantification methods such as ribonuclease assay, northern-blot, or competitive RT-PCR has the advantage of speed, throughput, and a high degree of potential automation (García-Fernández et al., 2009, Vandesompele et al., 2002). However, RT-PCR approaches require the same kind of normalization as traditional methods of mRNA quantification. In gene expression analysis, several variables need to be controlled such as the amount of starting material, differences between tissues or cells in overall

transcriptional activity, and enzymatic efficiencies (Hickford et al., 2010, Vandesompele et al., 2002). To normalize these variations, several strategies have been employed. For example, the gene transcript number is ideally standardized to the number of cells under controlled conditions of reproducible extraction of good quality RNA; however, exact enumeration of cells is often prevented when solid tissues are used (Schulze and Downward, 2001, Vandesompele et al., 2002).

In setting up a RT-qPCR study, one of the most disregarded points is experimental design. However, proper set-up of the experiment saves time, increases the accuracy and precision of the outcomes, and cuts down on reagent cost (Derveaux et al., 2010, Wong and Medrano, 2005). In general, there are 3 important aspects involved in experimental design:

- To draw meaningful and statistically significant outcomes, careful assessment of the number of biological samples is needed
- The proper run layout strategy should be selected
- The input RNA material should be free of contaminating DNA (Derveaux et al., 2010, Schulze and Downward, 2001, Vandesompele et al., 2002, Wong and Medrano, 2005).

Sample extraction and quality assurance

Good quality RNA is always required to produce reliable results. In the microarray field, the cost of experiments pushes people to access the purity and integrity of the input RNA. However, RT-qPCR is relatively cheap, and this might mislead an uninformed user; for example, the person might not realize the impact of RNA quality on the result (Derveaux et al., 2010, Vandesompele et al., 2002).

Housekeeping genes

Gene expression fields are central to realize the molecular basis of specific functional gene expressions in specific tissues and/or organisms in mammalian growth, development, and production. One of the most accurate methods to evaluate small changes in mRNA levels for individual genes is real-time PCR (qPCR). However, the quality of the outcomes is directly related to standardization/normalization with housekeeping/reference genes whose expression is stable (Barendse et al., 2009, Zang et al., 2011, Wang et al., 2009). According to Vandesompele *et al.* (2002), errors of up to 20-fold can result from using only one reference gene and at least two are recommended." He manifested the application of a single reference gene can result to aberrant gene expression values, and it is widely accepted that employing several reference genes for standardization/normalization is preferable (Vandesompele *et al.*, 2002).

Housekeeping or reference genes are generally constitutive genes that are transcribed at a relatively fixed level throughout various conditions, such as tissue type or developmental stage; also, their expression is presumed to be unaffected by experimental parameters (Zang et al., 2011, Vandesompele et al., 2002). Many studies demonstrated that no ideal universal reference gene exists, and even transcript levels of reference genes can vary considerably if reference genes are used without proper validation of their presumed stability of expression (Anderle et al., 2003, Asyali et al., 2006, Zang et al., 2011). Thus, the application of a single reference gene for normalization of gene expression studies should not be regarded as sufficient. The selection of desirable reference genes for normalization of qPCR data during development is by no way little; thus, they must be relatively unaffected by marked changes in transcriptional activity (Bagnall and Kotze, 2010, Zang et al., 2011). However, this task can be accomplished by computational methods lately developed to assess the expression stability of candidate

housekeeping genes, namely, NormFinder, geNorm, and BestKeeper. NormFinder utilizes a mathematical model to figure overall expression variation of candidate housekeeping genes and variation between sample groups, whereas geNorm employs a pair-wise analysis of gene expression to discover stable housekeeping genes. Similarly, BestKeeper performs a pair-wise comparison (Barendse et al., 2009, DeBusk, 2010, Zang et al., 2011).

Because there is not a single accepted method to analyse gene expression stability and different statistical methods can potentially give variable outcomes, validating the housekeeping genes utilizing several applications and identifying differences and similarities between the outputs of alternative software creates more reliable consensus decisions (Derveaux et al., 2010, Estelle et al., 2009, Zang et al., 2011).

Micro RNAs

MicroRNAs (miRNAs) are small regulatory molecules, endogenous non-coding RNAs, 18~25 nucleotides which are important in many biological processes and influence the expression of hundreds of genes (Jin *et al.*, 2010). These functionally important small RNAs were first reported in nematodes in 1993; nevertheless, researchers began to realize the function of microRNAs in 2001 (McDaneld *et al.*, 2009). miRNAs participate in the regulation of the gene expression of numerous biological processes, including lipid metabolism, organ development, brain morphogenesis, apoptosis, and differentiation (Li *et al.*, 2010a). They also can imperfectly bind target messenger RNAs (mRNAs) and inhibit translation by partially block expression of the target or target the mRNA for degradation or deadenylation (Zhou *et al.*, 2010). To date, across 133 species including primates, worms, viruses, plants, fish, rodents, birds, and flies, 14,197 miRNAs have been reported and described (Li *et al.*, 2010a). Although

miRNAs can regulate the expression of target genes by binding to complementary sites, they more regularly down-regulate the expression of target genes (Sheng *et al.*, 2011).

MicroRNA biogenesis

The biogenesis of miRNA is relatively well realized and has been reviewed (Liu *et al.*, 2010). MicroRNA are initially transcribed as long transcripts, called pri-miRNA, that can comprise either a single miRNA or a cluster of miRNAs (so-called pre-miRNA). The endonuclease Drosha is produced when pri-miRNA transcripts are processed into smaller hairpin structures (Trakooljul *et al.*, 2010). The matured miRNA sequence is preferentially loaded onto an RNA-protein complex known as the RNA induced silencing complex (RISC) during processing from the primary transcript (McDaneld *et al.*, 2009). The 3'-untranslated region (3'UTR) of the targeted mRNA is targeted by the miRNA-RISC complex, and then its expression is blocked by either degradation or sequestering it away from the ribosomal machinery (Trakooljul *et al.*, 2010). The association of RISC and mRNA in either case induces reduced translation of the targeted gene product. Consequently, miRNA have been reported to guide developmental decisions because of decreased translation of their cognate targets (McDaneld *et al.*, 2009). Thus, miRNAs are used to study cell cycle progression, adipocyte differentiation, cell fate, and processes that change muscle development and growth including skeletal muscle hypertrophy, myoblast proliferation, and differentiation (Liu *et al.*, 2010). Fundamentally, there are two sorts of approaches to identify miRNAs. One is done by sequencing size-fractionated cDNA libraries that many important miRNAs have been identified by this method (Sheng *et al.*, 2011).

miRNA control of posttranscriptional gene regulation

MicroRNA are small RNA that regulate gene translation; nevertheless, the mechanisms by which miRNA regulate this process remain controversial. By changing posttranscriptional regulation, miRNA have a function in leading developmental decisions, including cell cycle

progression, adipocyte differentiation and processes, cell fate, and apoptosis that change muscle development and growth (McDaneld et al., 2009, McDaneld, 2009, Nielsen et al., 2010). According to Jackson and Standart (2007), It has been well documented that miRNAs decrease translation of protein-coding genes (McDaneld, 2009). However, Buchan and Parker (2007) and Vasudevan (2007) state that controversy surrounds the proposed mechanisms of action. Evidence has also emerged that contradicts the current dogma of decreased gene translation (McDaneld, 2009).

Due to the increase in discovered miRNA sequences, a public database has been dedicated to catalogue predicted and experimentally detected miRNA (McDaneld et al., 2009, McDaneld, 2009, Nielsen et al., 2010) . To date, over 6,300 sequences have been submitted to MiRBase including livestock sequences. However, their function in biological processes has not been fully determined (Benes and Castoldi, 2010, McDaneld, 2009). miRNA genes are mostly located in intergenic regions. However, they have also been discovered in introns and exons of protein coding genes. In all studied species, transcription of miRNA genes is tightly regulated spatially between tissues and temporarily during development within tissues (Cho et al., 2010, McDaneld, 2009). Furthermore, multiple mechanisms including other miRNA, transcription factors, and epigenetic are regulated by miRNA genes (Jin et al., 2010, McDaneld, 2009). As an example, muscle-specific transcription factors, myoblast-de-termining protein, and myocyte-enhancing factor-2 have been demonstrated to be activators of muscle-specific miRNA, miR-1, and miR-133 transcriptions by an intragenic enhancer (Kim et al., 2008, Li et al., 2011, McDaneld, 2009).

Blocking of Initiation

An optional mechanism of posttranscriptional control by miRNA started to emerge as research found that mRNA abundance did not always decrease with gene translation and proposed that miRNA regulate translation through multiple mechanisms (Li et al., 2010a, Li et al., 2010b, McDaneld, 2009). The proposed theory states that miRNA binds to the RISC and targets the 3' UTR of the mRNA sequence. Thus, the miRNA:RISC complex to the 3' UTR is supposed to activate a sequence of a events that block initiation protein from binding to the 5' cap of the mRNA (Liu et al., 2010, McDaneld et al., 2009, McDaneld, 2009, Nielsen et al., 2010).

Translocation to Processing Bodies

This proposed mechanism of miRNA action requires posttranscriptional regulation by translocation of the miRNA::mRNA complex to cytoplasmic foci in the cell, recognized as processing bodies (P-bodies) after the miRNA:RISC complex binds to the mRNA target (McDaneld, 2009, Sheng et al., 2011).

Activation of Translation

In late 2007, the understanding of posttranscriptional regulation was challenged by reports stating that miRNA was also increasing gene translocation (McDaneld, 2009, Trakooljul et al., 2010, Wan et al., 2010).

Role of miRNA in production livestock

An emerging field of interest for animal scientists is the role of miRNA in cellular processes that affect animal biology. Priority research has centred on expanding the current miRNA database (miRBase) to include sequences for livestock species by homology searches and evaluation of miRNA transcriptome profiles (McDaneld, 2009, Trakooljul et al., 2010). With the increased number of miRNA sequences in the database, interest has expanded to determine

what role miRNA has in animal biology (McDaneld, 2009, Trakooljul et al., 2010). Therefore, it is important to review the current miRNA research for traits of value in livestock species to determine our current knowledge and the direction of future research.

Table 2.4 Significant miRNAs involved in fat metabolism and synthesis

miRNA	Tissue	Species	Effect	Reference
miR-103		Pig	brain development, lipid metabolism, adipocyte, differentiation, hematopoiesis, and immunity	(Li *et al.*, 2010a)
miR-499	Heart specific	Pig	Just significantly expressed	(Reddy *et al.*, 2009)
miR-208	Heart specific	Pig	Just significantly expressed	(Reddy *et al.*, 2009)
miR-122	Liver specific	Pig	Just significantly expressed	(Reddy *et al.*, 2009)
miR-1	Heart	Pig	Just significantly expressed	(Reddy *et al.*, 2009)
miR-133	Heart	Pig	Just significantly expressed	(Reddy *et al.*, 2009)
miR-181a	Thymus	Pig	Just significantly expressed	(Reddy *et al.*, 2009)
miR-142-3p	Thymus	Pig	Just significantly expressed	(Reddy *et al.*, 2009)
miR-194	Liver	Pig	Just significantly expressed	(Reddy *et al.*, 2009)
miR-143	stomach	Pig	Just significantly expressed	(Reddy *et al.*, 2009)
miRNA-143	marbling muscle tissue	Holstein steers	differentiation of bovine intramuscular fat, which in part contributes to the regulated expression of adipocyte genes.	(Li *et al.*, 2010b)
miR-143	ubiquitously expressed among tissues	Chicken	regulation of cell proliferation and apoptosis	(Trakooljul *et al.*, 2010)
miR-1826	longissimus muscle tissue	Pig	highly expressed at embryonic day 90	(Zhou *et al.*, 2010)
miR-26a	longissimus muscle tissue	Pig	highly expressed at embryonic day 90	(Zhou *et al.*, 2010)
miR-199b	longissimus muscle tissue	Pig	highly expressed at embryonic day 90	(Zhou *et al.*, 2010)
let-7	longissimus muscle tissue	Pig	highly expressed at embryonic day 90	(Zhou *et al.*, 2010)
miR-1a	longissimus muscle tissue	Pig	showed highest abundance during the fast growing stage at postpartum day 120	(Zhou *et al.*, 2010)
miR-133a,	longissimus muscle tissue	Pig	showed highest abundance during the fast growing stage at postpartum day 120	(Zhou *et al.*, 2010)
miR-26a	longissimus muscle tissue	Pig	showed highest abundance during the	(Zhou *et al.*, 2010)

miR-1826	longissimus muscle tissue	Pig	fast growing stage at postpartum day 120 showed highest abundance during the fast growing stage at postpartum day 120	(Zhou *et al.*, 2010)
miR-143	adipose tissue	Swine	the most abundant miRNAs	(Li *et al.*, 2011)
miR-103	adipose tissue	Swine	the most abundant miRNAs	(Li *et al.*, 2011)
let-7	adipose tissue	Swine	the most abundant miRNAs	(Li *et al.*, 2011)
miR-148	adipose tissue	Swine	the most abundant miRNAs	(Li *et al.*, 2011)

In livestock production, evaluation of regulatory factors that affect development and growth of economically important tissue such as adipose tissue and skeletal muscle is the major interest due to the profit margin gained by nutrition portioning between these tissues (McDaneld, 2009, Xie et al., 2010, Zhou et al., 2010). Although new sequencing technologies have enabled new miRNA sequences to be identified by deep sequencing of the miRNA transcriptome in chicken models, microRNA were initially reported to have a role in skeletal muscle development using zebrafish, mouse, and Drosophila models (McDaneld, 2009). In addition to skeletal muscle, the predominant research evaluating the role of miRNA has evolved in adipose tissue of human and mouse cell lines and later to miRNA transcriptome profiles in adipose tissues has been produced for cattle (McDaneld, 2009, Sheng et al., 2011).

Real-Time PCR and Real-Time RT-PCR applications in food labelling and gene expression studies

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Abstract

Polymerase chain reaction (PCR) as a scientific invention has revolutionized molecular biology and has led to real-time PCR and later to real-time reverse transcription PCR (Real-Time RT-PCR). These two techniques enable scientists to conduct PCR detection of amplified gene products and expression analysis of targeted genes. Quantitative polymerase chain reaction (qPCR), also called real-time polymerase chain reaction, is a recent modification to PCR that utilizes fluorescent reporter molecular techniques to monitor the production of amplified products during each cycle of the PCR reaction and enables both detection and quantification of specific sequences in complex mixtures. Over the past decade, real-time PCR applications have rapidly changed the nature of molecular science and become widely used tools in molecular genetics research. Real-time PCR permits specific, sensitive, and reproducible manipulation of nucleic acids by combining the nucleic acid amplification and detection steps using gel electrophoresis. Hence, it almost eliminates the need for DNA sequencing or Southern blotting for amplicon identification. One of the many versions of PCR is real-time

(RT-PCR), which has become one of the most broadly used gene amplification and expression methods in molecular biology research. Real-time RT-PCR is commonly employed to discover RNA expression levels through the creation of complimentary DNA (cDNA) transcripts from RNA, and it is frequently confused with real-time PCR. Food labelling provides very important information to help both producers and consumers to make informed choices about healthier and safer food. The process that information from a gene is used in the synthesis of a functional gene product is called gene expression. It enables scientists to decipher the functions of genes. Food labelling and gene expression are fundamental to studying the relationships between the human genome, nutrition, and health in a relatively new specialist field called nutritional genomics. Nutritional genomics is expected to revolutionize the way health professionals and dieticians treat people in the future. Thus, it is anticipated that the focus of nutritional genomics research will in the future shift to determining the right type of food for an individual based on his or her genomic compatibility. Therefore, nutritional genomics will be able to aid people in avoiding foods that are inappropriate matches that could potentially impact negatively on the individual's health. This paper reviews the importance and power of real-time PCR application in food labelling and nutritional genomics, types of fluorescent-based chemistry procedures developed for real-time PCR detection, real-time RT-PCR application in gene expression studies, and the great potential of combining these technologies for animal molecular genetics research in sheep and fish.

Keywords: PCR, Real-Time PCR, Real-Time RT-PCR, Gene Expression

Introduction

Kary Mullis in 1984 made a revolution in science by inventing the polymerase chain reaction (PCR) (Deepak *et al.*, 2007). Polymerase chain reaction utilizes a pair of primers, each hybridizing to one strand of a double-stranded DNA target, with the pair spanning a region that is exponentially reproduced. The hybridized primer creates a complimentary strand through a sequential addition of deoxynucleotides (Fraga *et al.*, 2008; Mackay *et al.*, 2002). This process is summarized in the following steps: (i) at > 90 °C, the double-stranded DNA is separated; (ii) at 50-75 °C primer annealing occurs; and (iii) at 72-78 °C optimal extension is achieved as depicted in Fig 2.1 (Fajardo *et al.* 2010; Fraga *et al.*, 2008; Mackay *et al.* 2002).

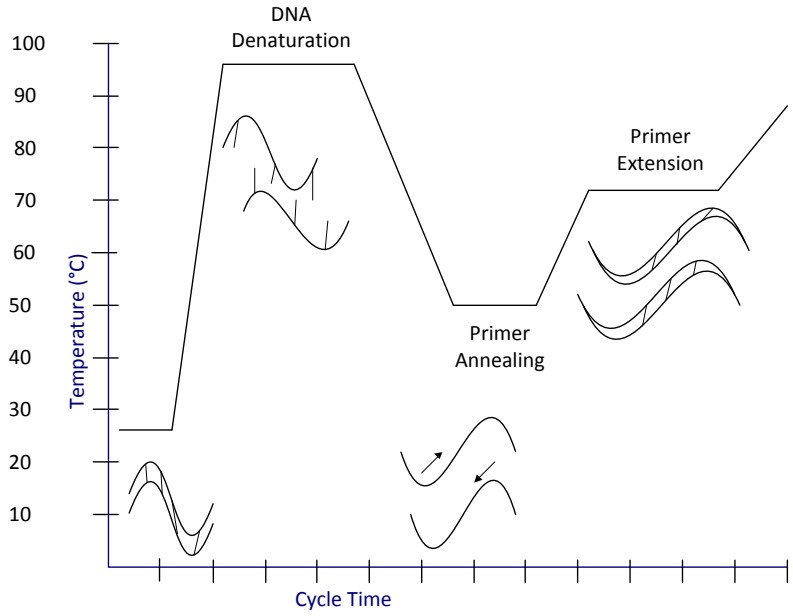

Figure 2.1 The PCR cycle

Some of the gold standards such as cell culture and serological assays have been displaced by the introduction of conventional PCR, which is used to obtain quantitative data with promising results (Deepak *et al.*, 2007; Fraga *et al.*, 2008; Mackay *et al.*, 2002). However, electrophoresis of the nucleic acids using ethidium bromide and visual or densitometric analysis of the resulting bands after irradiation by ultraviolet light are required for traditional detection of amplified DNA (Hoffmann *et al.*, 2009; Mackay *et al.*, 2002; Ponchel *et al.*, 2003)

In contrast to conventional assays, the theory of the detection of amplicons by visualization as the amplification progresses is highly regarded (Fraga *et al.*, 2008; Mackay *et al.*, 2002). This modification to PCR is called real-time or quantitative polymerase chain reaction (qPCR) (Fraga *et al.*, 2008; Schefe *et al.*, 2006), and was first introduced by Higuchi and colleagues in 1992 (Fraga *et al.*, 2008). Real time quantitative polymerase chain reaction permits accurate detection and quantification of specific nucleic acids in a complex mixture (Fraga *et al.*, 2008; Shiao, 2003). In real-time PCR, the procedure follows the general principles of PCR, and it is achieved by monitoring the amplification of a target sequence using fluorescence (Fraga *et al.*, 2008; Mackay *et al.*, 2002).

Over the past decade, real-time PCR has become a common and broadly used tool for detecting and quantifying expression profiles of selected genes by coupling it with reverse transcription (Bustin *et al.*, 2005; Deepak *et al.*, 2007; Fraga *et al.*, 2008). By the combination of these two technologies (reverse transcription polymerase chain reaction and real-time RT-PCR), the quantitative measurement of RNA transcription levels is accomplished (Deepak *et al.*, 2007; Fraga *et al.*, 2008; Shiao, 2003).

This paper's objective is to review published literature on the applications of real-time PCR and real-time RT-PCR in food labelling and gene expression studies in sheep and fish.

Real-Time PCR Applications

The Food Industry

In the modern diet, food labelling regulations require accurate declaration of product ingredients. This has become necessary because food adulteration is a common problem in the

processed foods industry. Adulteration occurs in the beef industry through the addition of minced meats (Köppel *et al.*, 2011; Tanabe *et al.*, 2007), just as much as the addition of mixed milk to dairy products occurs in the dairy industry (López-Calleja *et al.*, 2007). In order to ensure food safety for consumers who may have specific food allergies and to gain consumers' trust as well as minimize possible economic losses, several analytical methods have been developed to verify food labelling statements (Köppel *et al.*, 2011; López-Calleja *et al.*, 2007; Tanabe *et al.*, 2007).

In the past few years, protein-based methods and DNA analysis have been used to correctly label food products. However, protein-based methods have had limited success in cooked meat products due to protein denaturation during processing heat and pressure (Tanabe *et al.*, 2007; López-Calleja *et al.*, 2007). On the other hand, due to the high stability and unique variability of DNA molecules, they have been used as target compounds for species identification (Köppel *et al.*, 2011; Tanabe *et al.*, 2007). Among DNA-based methods, PCR techniques are highly accurate and relatively fast, hence their successful applications in the meat and dairy industries (Köppel *et al.*, 2011; López-Calleja *et al.*, 2007; Tanabe *et al.*, 2007). However, conventional PCR methods appear to lack quantitative capabilities.

In the recent past, the need for methods giving quantitative results has grown following the introduction of labelling obligations made by Authorized Food Control Agencies (Köppel *et al.*, 2011; Tanabe *et al.*, 2007). Among PCR-based methods that monitor the exponential amplification of target-specific DNA, real-time PCR has demonstrated the highest improvement in increased fluorescence signal (Kesmen *et al.*, 2012; Köppel *et al.*, 2011; Tanabe *et al.*, 2007).

A number of studies have successfully developed real-time PCR methods for the food and dairy industries. For example, TagMan-based real-time PCR methods for the detection and separation of chicken from turkey meat (Kesmen *et al.*, 2012) and distinguishing between pork, chicken, beef, mutton, and horse meat (Tanabe *et al.*, 2007) have been published. Furthermore, multiplex real-time PCRs have been developed for the quantification of DNA from beef, pork, horse, and sheep (Köppel *et al.*, 2011), and real-time quantitative PCR assays for the detection of goat milk in sheep milk have also been developed (López-Calleja *et al.*, 2007) (Table 2.5).

Table 2.5 The application of real-time PCR in food labelling industry

Study	**Sample**	**Reference**
Detection of chicken and turkey meat	Raw and cooked meat	(Kesmen et al., 2012)
Detection method for distinguishing pork, chicken, beef, mutton, and horse flesh	Food	(Tanabe et al., 2007)
Multiplex real-time PCR for the detection and quantification of DNA from beef, pork, horse and sheep	Raw and cooked meat	(Köppel et al., 2011)
Quantitative detection of goat milk in sheep milk by real-time PCR	Milk	López-Calleja *et al.*, 2007)

The tremendous utility and diversity of possible areas of application of real-time PCR techniques make it affordable for most laboratories and industries to use in research and food inspection programs.

Nutritional Genomics

In line with increasing world population, meat and milk consumption is predicted to increase globally. The risk of cardiovascular disease, some cancers, and diabetes has been shown to be related to dietary intake of unsaturated fatty acids (UFA) (Mutch *et al.*, 2005; Woods and Fearon, 2009). In addition, animal products have been criticized for their high content of saturated fatty acids (SFA). Therefore, the nutritional modification of animal diets to increase the proportion of UFA at the expense of SFA has been the subject of continuous research interest in meat, milk, and eggs (Mutch *et al.*, 2005; Woods and Fearon, 2009).

Nutrients can interact with and modulate the molecular mechanisms of physiological functions in an organism. Erroneous scientific conclusions and misinformed nutritional recommendations have resulted from performing population–scale epidemiological studies in the absence of genetic knowledge (Mutch *et al.*, 2005). To overcome such issues and to understand more about the relationship between genes and diets, the field of nutrition has begun to introduce technologies and supporting analytical software to elucidate the interactions between diets and genes (Mutch *et al.*, 2005; Woods and Fearon, 2009).

Nutritional genomics is divided into two disciplines: nutrigenomics and nutrigenetics. Nutrigenomics examines the effect of nutrients and dietary ingredients on health by altering the genome, proteome, and metabolome. In this regard, there are scientific attempts to link the resulting phenotypic variation to differences in the genetic response of the whole biological system. On the other hand, nutrigenetics analyses the effect of genetic variations on the interaction between diet and health. It aims to investigate how the genetic constitution of an individual coordinates their response to diet, and hence it studies the underlying genetic polymorphisms (Fenech *et al.*, 2011; Mutch *et al.*, 2005)

As an example, a nutrigenetics study related to cardiovascular disease and cancer was conducted in Singapore (Fenech *et al.*, 2011). Singapore's population includes large segments of diverse ethnic backgrounds, comprising Malays, Chinese, and Indians, and it has undergone a very rapid socio-economic development (Fenech *et al.*, 2011). Due to this rapid development, life expectancy has risen, and mortality figures have changed from malnutrition and infectious diseases to cardiovascular disease and cancer, which now represent the top two causes of death in Singapore (Fenech *et al.*, 2011). Living in this ethnic heterogeneity as an integrated community within a homogenous environment provided an interesting opportunity to study the impact of ethnicity on cardiovascular and cancer disease throughout the rapid economic transition (Fenech *et al.*, 2011).

The results revealed that in relation to metabolic and cardiovascular diseases, the epidemiological transition had not affected all ethnic groups equally. The study indicated that Singaporeans of Indian origin appeared to be at the highest risk of susceptibility to cardiovascular disease, followed by the intermediate rate in Malays, and the lowest risk in Chinese (Fenech *et al.*, 2011).

Fluorescence-Based Chemistry Versus SYBR-Green

One of the main problems of research within a clinical diagnostic setting is the detection and quantification of gene re-arrangement, amplification, translocation, or deletion (Fraga *et al.*, 2008; Ponchel *et al.*, 2003). However, real-time PCR has become a well-established procedure for quantifying the levels of gene expression.

There are several fluorescent-based chemistry procedures developed for real-time PCR detection, which are classified into four main types by Fajardo *et al.* (2010):

- Hydrolysis probe (e.g., TagMan)
- Hairpin probes (e.g., molecular beacons)
- Fluorescent labelled hybridization probes (e.g., FRET)
- DNA intercalating dyes.

Hydrolysis Probes

Hydrolysis probes are designed to increase the specificity of real-time PCR assays and are represented by TagMan chemistry principles. The principle relies on the 5'-3' exonuclease activity of fluorescence upon probe hydrolysis to detect PCR product accumulation (Fraga *et al.*, 2008; Wong and Medrano, 2005). The TagMan probe is labelled with fluorophore and covalently attached to the 5' end, and a quencher dye is attached to the 3' end (Wong and Medrano, 2005). Similar to other PCR methods, the resulting fluorescence signal allows the quantitative measurement of product accumulation during the exponential stages of PCR. However, the specificity of detection is significantly increased by the TagMan probe (Fraga *et al.*, 2008; Wong and Medrano, 2005).

Hairpin Probes

The presence of specific nucleic acids in homogenous solutions is often reported by oligonucleotide hybridization probes also known as molecular beacons (Fraga *et al.*, 2008; Wong and Medrano, 2005). Molecular beacons are the simplest among hairpin probes and consist of a loop-shaped region of sequence-specific primers flanked by two inverted repeats (Fraga *et al.*, 2008; Wong and Medrano, 2005). Molecular beacons represent a non-radioactive method for detecting specific sequences of nucleic acids (Fraga *et al.*, 2008; Wong and Medrano, 2005).

Fluorescent Labelled Hybridization Probes

Hybridization probes are fragments of DNA or RNA used to detect the presence of nucleotide sequences that are complementary to the sequence in the probe (Wong and Medrano, 2005). Hybridization probes can be used in either a three- or four-oligonucleotide assay. In the four-oligonucleotide method, PCR primers and two sequence–specific probes bind next to each other in a head-to-tail arrangement (Fraga *et al.*, 2008; Wong and Medrano, 2005). To ensure hybridization of the probe to its target sequence, the probe is tagged as a radioactive or fluorescent molecular marker (Fraga *et al.*, 2008; Wong and Medrano, 2005). Hybridization probes are used in the field of microbial ecology in order to determine the presence of microbial species, genera, or microorganisms (Fraga *et al.*, 2008; Wong and Medrano, 2005).

DNA Intercalating Dyes

DNA intercalating dyes start emitting fluorescence when bound to double-stranded DNA (dsDNA). During PCR cycling, the double-stranded PCR product accumulates more dye, binds and emits fluorescence (Fraga *et al.*, 2008; Wong and Medrano, 2005). Hence, the fluorescence intensity increases proportionally to dsDNA concentration. In this technique, one dye can be used for different gene assays, which makes it very flexible (Fraga *et al.*, 2008; Wong and Medrano, 2005).

In probe-based chemistry, real-time PCR assays allow the use of very small amplicons and internal probes which bind to the desired specific sequence within the amplicon. As a result, specific and efficient amplification is accomplished (Fajardo *et al.*, 2010). However, the possible limitations of these assays include the design and availability of appropriate primers and probes that must adapt to fixed PCR conditions. The most direct, simple, and inexpensive fluorescent system developed for real-time PCR is SYBR Green I, and it is based on the binding of the fluorescent dye unto the PCR product (Fajardo *et al.*, 2010; Ponchel *et al.*, 2003).

SYBR Green I

SYBR Green I is an asymmetrical cyanine dye. It involves the incorporation of the SYBR Green I dye, whose fluorescence under ultraviolet rays greatly increases when it is bound to the minor groove of the double helical DNA. It is used as a nucleic acid stain in molecular biology (Fajardo *et al.,* 2010). The advantage of using SYBR Green I dye is that it allows the interpretation of DNA melting curves in order to distinguish the specific amplicon of the PCR product from false positive signals resulting from non-specific amplification or primer dimers (Fajardo *et al.*, 2010; Fraga *et al.*, 2008; Etienne *et al.*, 2004).

Real-Time RT-PCR Method for Quantifying Gene Expression

During the last century, advancements in bioscience have assisted in our comprehensive understanding of the molecular mechanisms of phenotypic expression of genotypes (Bustin *et al.*, 2005; Deepak *et al.*, 2007). However, knowledge of the functions of a major part of the genome is still limited or unknown, and the relationship between enzymes, small molecules, and signalling substances is quite limited (Deepak *et al.*, 2007; Shiao, 2003). Gene expression analysis can yield valuable clues about gene function; thus it has been widely used to identify the type of cells or tissues where a gene is expressed (Fraga *et al.*, 2008), detecting the relationship between ecologically influenced or a defined biological state (e.g., development, differentiation, disease), cellular expression patterns (Deepak *et al.*, 2007), and discovering a change in gene expression level in response to a specific biological stimulus (e.g., pharmacological agent or growth factor) (Fraga *et al.*, 2008).

Conventional RT-PCR was widely used for gene-specific mRNA quantification during the early days of the introduction of real-time RT-PCR (Shiao, 2003). This method is called quantitative endpoint RT-PCR analysis. It is not able to determine the initial quantity of template molecules for a gene-specific PCR, due to reliance on measuring the end point of a

PCR reaction by ethidium bromide visualization of the DNA product separated by gel electrophoresis (Bustin *et al.*, 2005; Fraga *et al.*, 2008; Shiao, 2003). The amount of amplicon at the end of amplification cycles depends on the input amount, variable kinetics of the PCR reaction, and technical variations occurring during the reaction time frame (Bustin *et al.*, 2005; Etienne *et al.*, 2004). This variability is shown in Figure 3.2 compared to real-time RT-PCR which improves upon endpoint RT-PCR by measuring target amplification early in the reaction while amplification is continuing most efficiently (Deepak *et al.*, 2007; Etienne *et al.*, 2004; Fraga *et al.*, 2008).

Comparison of Gene Expression Analysis between Real-Time RT-PCR and Northern Blot Analysis and RNase Protection Assays

There are major advantages of using real-time PCR compared to conventional semi-quantitative PCR. Gene expression analysis using northern blot technique and RNase protection assays is time consuming, and it also difficult to reliably generate accurate measurements of gene expression levels (Deepak *et al.*, 2007; Etienne *et al.*, 2004; Fraga *et al.*, 2008). The application of real-time RT-PCR extends several advantages over the other methods, including requiring small amounts of sample to start, its power to reproduce rapid and accurate data, and its capacity for analysing multiplex genes at a time (Fraga *et al.*, 2008; Schefe *et al.*, 2006).

To work with extremely low amounts of mRNA, real-time RT-PCR is the technique of choice. By using an internal control, RT-PCR ensures normalization of the differences in the kinetics of the reverse transcription process, and adequately replaces other conventional methods (Fraga *et al.*, 2008; Shiao, 2003). In addition, multiplex real-time RT-PCR is desirable to examine multiple gene identification based on the utilization of fluorochomes and analysis of melting curves of the amplified products (Deepak *et al.*, 2007).

Real-time RT-PCR technology is now available for many livestock species, and animal researchers are beginning to use the technology to address issues of importance to animal agriculture (Table 2.6).

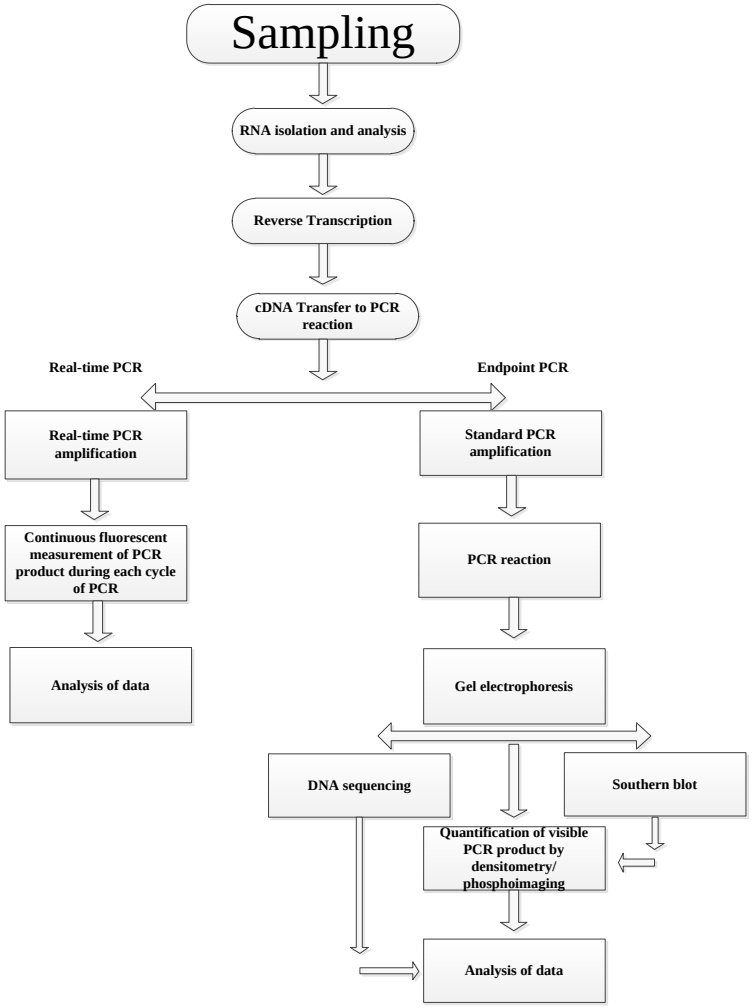

Figure 2.2 Comparison of end-point RT-PCR and real-time RT-PCR.

Discussion

Since the invention of PCR in 1984, there has been modification to the process of amplifying DNA and an evolution in the field of gene expression studies. To our knowledge, documentation and systematic review of how the PCR process has evolved remains scanty; hence there is a need for this review to provide the scientific community with the current applications of PCR and RT-PCR techniques. In the past, the real-time RT-PCR technique was

largely used for gene expression analysis in microorganisms, but it is now being increasingly applied in nutritional genomics to study ruminants, porcines, and fish (Fraga *et al.*, 2008; Kesmen *et al.*, 2012). For example, in a study of Euryhaline fish (Lates calcarifer), gene expression analysis revealed that dietary oils lacking long-chain polyunsaturated fatty acids (LC-PUFA) up-regulated key LC-PUFA biosynthesis genes in the muscle and liver tissues (Alhazzaa *et al.*, 2011). In a similar study, RT-PCR was employed to examine the gene expression of n-3 LC-PUFA biosynthesis in the white muscle and liver of Atlantic salmon (Codabaccus *et al.*, 2011). In this study, RT-PCR analysis demonstrated that Echium oil-fed fish had higher n-3 LC-PUFA levels in both liver and white muscle tissues compared to rapeseed oil-fed fish (Table 2.6) (Codabaccus *et al.*, 2011).

Gene expression analysis using real-time RT-PCR has been employed for studying the differences in inter-species relationships in veterinary medicine, hence, the need for conducting research in specific animal species to obtain accurate information of the constitutive expression of multi-drug transporters (Ballent *et al.*, 2013). Another example is the gene expression and transport efflux activity of P-glycoprotein in sheep liver and small intestines (Table 2.6) (Ballent *et al.*, 2013).

Real-time RT-PCR is a powerful tool for analysing mRNA expression of a gene(s) in different species in order to investigate the existence of homology or further phylogenetic relationships, for example, Rab-related protein Rab-2A (Rab2A), Rab-related protein Rab-3A (Rab3A), and Rab-related protein Rab-7A (Rab7A) genes in black-boned sheep (He *et al.*, 2010), Lymphocyte-specific protein tyrosine kinase (LCK) and Cyclin-dependent kinase 2 (CDK2) genes from the black-boned sheep (Yu *et al.*, 2010), and Sideroflexin 1 (Sfxn1), Snai homolog

2 (Snai2), and Protein cappuccino homolog (Cno) genes from black-boned sheep (Table 3.2) (Xi *et al.*, 2011).

Conclusion

Real time quantitative polymerase chain reaction is a unique and powerful tool for the detection of mRNA and gene expression levels in various research fields. Despite its advantages, the extreme sensitivity of this technique can be a drawback. A slight DNA combination can lead to undesirable results. Also, planning and designing of quantification studies can be technically challenging. Furthermore, the application of Real-time RT-PCR in nutritional studies including n-3 and n-6 fatty acid syntheses is still limited. Nevertheless, it is predicted that real-time RT-PCR as a technique for investigating gene expression in nutritional genomics research will continue to grow.

In the Animal Science and Genetics Research Laboratory at the University of Tasmania, Australia, the real-time RT-PCR technique is utilised to identify expression profiles of genes that affect fat and protein biosynthesis and metabolism in the liver, heart, muscle, kidney, and adipose tissues in genetically divergent purebred and crossbred Australian sheep supplemented with unconventional dietary protein sources like the microalga called *Spirulina*. We aim to trace the expression profiles of fat-related genes in different sheep tissues. This would allow us to determine a balanced level of dietary protein from gene expression data to increase the n-3 and n-6 contents of red meat among Australian crossbred sheep. We hope that this review of published literature on the applications of real-time PCR and real-time RT-PCR will give the scientific community systematic and updated information on the evolution and application of PCR in gene expression and food labelling studies.

Table 2.6 Applications of real-time RT-PCR technology in animal research.

Purpose	Species	Tissue	Gene	Reference
P-glycoprotein in sheep liver and small intestine gene expression and transport efflux activity	Sheep	Liver	ABCB1	Ballent et al., 2013
Analysis on cDNA sequence, mRNA expression and imprinting status of Dlk1 gene in goats	Sheep	Various parts	Dlk1	Cao et al., 2010
The quantification of prion gene expression in sheep using real-time RT-PCR	Sheep	neocortex, cerebellum, thalamus, obex, hippocampi, conarium and spinal cord	PrP	Han et al., 2006
Molecular cloning, sequence characterization and tissue transcription profile analyses of two genes: LCK and CDK2; from Black-boned sheep	Sheep	Spleen, skin, kidney, lung, liver & heart	LCK & CDK2	(Yu et al., 2010)
Molecular cloning, sequence identification and tissue expression profile of three novel gene S*fxn1*, *Snai2* and *Cno* from Black-boned sheep	Sheep	Leg muscle, kidney, skin, *Longissimus dorsi* muscle, spleen, heart & liver	Sfxn1, Snai2 & Cno	Xi et al., 2011
Isolation, sequence identification and expression profile of three novel genes *Rab2A*, *Rab3A* and *Rab7A* from black-boned sheep	Sheep	Leg muscle, kidney, skin, *Longissimus dorsi* muscle, spleen, heart & liver	Rab2A, Rab3A & Rab7A	He et al., 2010
An extended feeding history with a stearidonic acid enriched diet from parr to smolt increase n-3 long-chain polyunsaturated fatty acids biosynthesis in white muscle and liver of Atlantic salmon (*Salmo salar* L.)	Atlantic salmon	Muscle & liver	Δ6 desaturase, Δ5 desaturase, elongase	Codabaccus et al., 2011
Up-regulated desaturase and elongase gene expression promoted accumulation of polyunsaturated fatty acid (PUFA) but not long-chain PUFA in *Lates calcarifer*, a tropical euryhaline fish fed a stearidonic acid-and γ-linoleic acid-enriched diet	Fish	Liver & skeletal muscle	Ubq, EF1-α, β-actin, FAE & FAD6	Alhazzaa et al., (2011)

Chapter 3

Effect of dietary supplementation with *Spirulina on* the expression of Aralkylamine N-acetyltransferas, Adrenergic beta-3 receptor, B-cell translocation gene *and* Fatty acid synthase genes in the subcutaneous adipose and muscle tissues of crossbred Australian sheep

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Abstract

Background

The demand for healthy, lean, and consistent meat products containing low saturated fatty acid content and high quality polyunsaturated fatty acids (PUFA), especially long-chain (≥C20) n-3 PUFA, has increased in recent times. Fat deposition is altered by both the genetic background and dietary supplements, and this study aimed to assess the effect of dietary Spirulina supplementation levels on the mRNA expression patterns of genes controlling lipid metabolism in the subcutaneous adipose tissue (SAT) and longissimus dorsi (ld) muscle of Australian crossbred sheep.

Methods

Twenty-four weaned lambs belonging to four breeds under the same management conditions were maintained on ryegrass pasture and fed three levels of Spirulina supplement (0, 10, or 20%). After slaughter, SAT and ld samples were subjected to mRNA extraction and reverse transcription using quantitative polymerase chain reaction (RT-qPCR) to assess the mRNA expression levels of the Aralkylamine N-acetyltransferase (AANAT), Adrenergic beta-3 receptor (ADRB3), B-cell translocation gene 2 (BTG2), and Fatty acid synthase (FASN) genes, which are associated with lipid metabolism.

Results

Both the 10 and 20% Spirulina supplementation levels strongly up-regulated the transcription of all the selected genes in both SAT and ld tissues (mostly in the subcutaneous adipose), but sheep breed and sex did not influence the gene expression patterns in these tissues.

Conclusions

The evidence indicates that 20% Spirulina supplementation resulted in a decrease in intramuscular fat content in Australian crossbred sheep due to the enhanced production of melatonin in sheep muscle tissues and strong up-regulation of mRNA expression of BTG2 in SAT, which negatively affected fat deposition. In contrast, 10% Spirulina supplementation strongly up-regulated the ADRB3 and FASN genes responsible for fat production. These findings are consistent with the observed phenotypic data suggesting that 10% Spirulina supplementation level can increase lamb production with richer long-chain PUFA content.

Background

Inclusion of marine supplements in the diet of sheep represents an effective nutritional strategy for altering meat production and enhancing polyunsaturated fatty acids (PUFA) (Bichi et al., 2013, Holman et al., 2012). *Spirulina* (*Arthrospira platensis*), is an edible blue-green microalga

and a highly nutritious food containing 60 to 70% protein for humans and is a potential feed resource for many animals. Research findings have linked *Spirulina* to an improvement in animal growth and nutritional product quality (Holman et al., 2012, Holman et al., Holman and Malau-Aduli, 2013).

Meat with superior eating qualities and healthier nutritional composition demands a higher price, and consumers are generally prepared to pay this higher price for higher quality meat. Traditionally, the fat content of meat has been considered important as a source for essential fatty acids and as a calorie dense nutrient (Guo *et al.*, 2014). During the several decades, fats and particularly the fatty acids component have been increasingly recognised as major biological regulators that affect the technological quality of fresh meat and the sensory quality of meat products (Deckelbaum et al., 2006, Perez et al., 2010). In addition, fatty acids can influence sterol metabolism, signal transduction, enzyme activities, cell proliferation and differentiation, and receptor expression (Deckelbaum et al., 2006, Guo et al., 2014). The effects of fatty acids in cell biology relate to their abilities to regulate gene expression and subsequent downstream events, and n-3 fatty acids are specially potent at affecting many of the above pathways (Deckelbaum *et al.*, 2006).

Fat deposition and composition can be altered by both genetic background and dietary supplements, which indicate their influence on adipogenic and lipogenic factors (da Costa *et al.*, 2013). However, the molecular mechanisms underlying fat deposition and fatty acid composition in sheep are not yet fully understood. To our knowledge, there is only limited available information that addresses the molecular adaptation of ovine tissues to supplementation with marine ingredients in the diet, which induces meat production and PUFA enhancement.

This study aimed to investigate changes in the mRNA expression patterns of the key genes *Aralkylamine N-acetyltransferase* (*AANAT*), *Adrenergic beta-3 receptor* (*ADRB3*), *B-cell translocation gene 2* (*BTG2*), and *Fatty acid synthase* (*FASN*) controlling lipid metabolism in subcutaneous adipose tissue (SAT) and *longissimus dorsi* (*ld*) muscle of Australian crossbred lamb under the effect of dietary *Spirulina* levels.

Methods

Animal and data collection

Use of animals and procedures performed in this study were approved by the University of Tasmania Animal Ethics Committee and were conducted in accordance with the 1993 Tasmanian Animal Welfare Act and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The design of the experiment has been described previously (Holman *et al.*, 2012). Briefly, 24 crossbred lambs were randomly assigned to four groups. Four groups of animals received 0, 10 and 20% *Spirulina* supplements, respectively. Following slaughter, 10 g of subcutaneous adipose and muscle tissues were collected from the carcass for RNA extraction and further analysis.

RNA extraction and cDNA synthesis

Total RNA was isolated from frozen tissue using TRIzol® Plus RNA Purification Kit (Life Technologies Pty Ltd. Victoria, Australia). Homogenisation of the sample in TRIzol®

Reagent was performed using a tissue lyser (Qiagen Ltd., Crawley, UK), and the RNA was subsequently extracted using chloroform and precipitated using isopropanol. Quantity and quality of total RNA was assessed using the NanoDrop 8000 spectrophotometer (NanoDrop, Wilmington, DE), RNA quality was verified by ensuring all RNA samples had an absorbance (A260/280) of between 1.8 and 2. RNA samples were treated with PureLink™DNase (Life

Technologies Pty Ltd. Victoria, Australia) and purified using the RNeasy1 Mini Kit (Qiagen Ltd.). DNase-treated and purified total RNA was then reverse transcribed to cDNA with Mixed Oligo dT/Random Hexamer Primers using the Tetro cDNA Synthesis Kit (Bioline Pty Ltd. NSW, Australia) according to the manufacturer's instructions and stored at -80 °C for subsequent analyses.

Primer design and reference gene selection

All candidate and reference gene primers used to detect the gene expression (Table 3.1) in this study were designed using the Primer3 web based software program (http://frodo.wi.mit.edu/primer3/), and they were obtained from a commercial supplier (GeneWorks Pty Ltd., SA, Australia). Primer specificity was checked using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). All of the primers were validated using a pooled cDNA sample. A standard curve was generated using serial dilutions of pooled cDNA. Polymerase chain reaction products generated by amplification were sequenced to verify their primer-specific identity (Beckman Coulter CEQ^{TM} 8000 Series Genetic Analysis System, University Tasmania). To determine the relative gene expression levels, suitable highly stable reference genes were required. In the current study, of a total of five reference genes tested, two [*Ubiquitin C* (*UBC*) and *Peptidyl-prolyl cis-trans isomerasa* (*PPIA*)] were used to normalise gene expression data for *AANAT*, *BTG2*, *FASN*, and *ADRB3* transcript levels in both tissue types. The principle behind the selection of the reference gene is that the expression ratio of two perfect reference genes should be constant across all samples. The expression stability of the reference genes was validated using geNorm (version 3.5) software by calculating the gene expression stability measure (M value).

Quantitative real-time PCR (qPCR)

Following reverse transcription, the cDNA quantity was determined and standardised to the required concentration for qPCR. Triplicate 20 μL reactions were carried out in a 72-well Rotor-Gene (QIAGEN GmbH, Hilden, Germany) containing 4 μL cDNA (50 ng), 10 μL 2× SensiFAST SYBR No-ROX Mix (Bioline Pty Ltd., NSW, Australia), 4.4 μL DEPC H_2O, and 0.8 μL forward and reverse primers (100 fmol). Assays were performed using the Rotor-Gene 3000 (QIAGEN Pty Ltd., VIC, Australia) with the following cycling parameter: 95°C for 2 min polymerase activation; 40 cycles of 95°C for 5 s denaturation, 60°C for 10 s annealing, 72°C for 5 s extension. Gene expression levels were recorded as Ct values (i.e., the number of PCR cycles at which the fluorescence signal is detected above the threshold value), and all samples were run in triplicate. Amplification efficiencies were determined for all candidate and reference genes using the formula E=10^(−1/slope), with the slope of the linear curve of cycle threshold (Ct) values plotted against the log dilution (Higuchi *et al.*, 1993). Primer concentrations were optimised for each gene, and disassociation curves were examined for the presence of a single PCR product. The efficiency of the reaction was calculated using a 5-fold serial dilution of cDNA and generation of a standard curve. All PCR efficiency coefficients were between 1.7 and 1.8, and therefore they were deemed acceptable. The software package Rotor-Gene 3000 (version 6.0.16) (QIAGEN Pty Ltd., VIC, Australia) was used for efficiency correction of the raw Ct values, inter-plate calibration based on a calibrator sample included on all plates, averaging of replicates, normalisation to the reference gene, and the calculation of quantities relative to the highest Ct and log2 transformation of the expression values for all genes.

Statistical Analysis

A generalised linear model (GLM) (SAS Inst., NC) was used to compute the fixed effects of *Spirulina* supplementation, sire-breed, and sex, and their interactions on mRNA expression level of *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes in subcutaneous adipose and muscle tissues.

Results

Spirulina supplementation and phenotypic data

Spirulina supplementation enabled sheep to grow longer bodies (BL) than the control group (*P<0.015*). Furthermore, lambs in the 20% *Spirulina* supplementation treatment group had greater body condition score (BCS) than the 10% and 0% (control) treatment groups (*P<0.001*). It was observed that sheep receiving 10% *Spirulina* supplementation had the heaviest body weight (BWT) of 41.9 kg (*P<0.018*), however, no differences between the 20% and control treatment groups were observed. The phenotypic results are shown in Table 3.1.

Table 3.1 Primer pairs designed for real-time PCR (qPCR)

[a]Aralkylamine N-acetyltransferase=AANAT, β3-adrenergic receptor=ADRB3, B-cell translocation gene 2=BTG2, Fatty acid synthase=FASN, Ubiquitin C=UBC, Peptidyl-prolyl cis-trans isomerasa=PPIA, [b]Ta=Empirical annealing Temperature.

[a]Gene symbol	qPCR Primers		[b]T_a	Amplicon Size (bp)
	Forward Primer	Reverse Primer		
AANAT	ACTGACCTTCACGGAGATGC	TTCACTCATTCTCCCCGTTC	60	211
ADRB3	TCAGTAGGAAGCGGGTCGGG	GGCTGGGGAAGGGCAGAGTT	60	291
BTG2	CTGGAGGAGAACTGGCTGTC	AAAACAATGCCCAAGGTCTG	60	194
FASN	GTGTGGTACAGCCCCTCAAG	ACGCACCTGAATGACCACTT	60	110
Reference genes				
UBC	CGTCTTAGGGGTGGCTGTTA	AAATTGGGGTAAATGGCTAGA	60	90
PPIA	TCATTTGCACTGCCAAGACTG	TCATGCCCTCTTTCACTTTGC	60	72

Table 3.2 Least square means (LSM) of chest girth, wether height, body length, body condition score, live weight and average daily gain in *Spirulina* supplemented crossbred lambs

	Spirulina			**Breed**				**Sex**		**P Values**		
	0	10%	20%	BS	WS	D	M	W	E	*Spirulina*	Breed	Sex
GC (cm)	95.0	95.6	96.1	99.0^{a}	94.4^{b}	93.8^{b}	95.0^{b}	96.2^{a}	94.9^{b}	0.376^{ns}	0.001***	0.034*
WH (cm)	62.9	62.7	63.1	63.6^{a}	62.8^{a}	63.5^{a}	61.6^{b}	63.4^{a}	62.4^{b}	0.669^{ns}	0.001***	0.009**
BL (cm)	65.7^{b}	66.6^{a}	66.8^{a}	68.8^{a}	67.0^{b}	66.9^{b}	62.6^{c}	66.5	66.2	0.015*	0.001***	0.269^{ns}
BCS (0-5)	3.2^{b}	3.3^{b}	3.4^{a}	3.7^{a}	3.3^{b}	3.2^{b}	3.1^{c}	3.3	3.3	0.001***	0.001***	0.346^{ns}
BWT (kg)	40.6^{b}	41.9^{a}	40.8^{b}	46.3^{a}	42.9^{b}	41.8^{b}	33.5^{c}	42.1^{a}	40.1^{b}	0.018**	0.001***	0.001***
ADG (kg/d)	0.1	0.2	0.1	0.1	0.2	0.2	0.1	0.1	0.1	0.759^{ns}	0.502^{ns}	0.605

Column means within a fixed effect bearing different superscripts significantly differ (P<0.05). Chest girth (CG), withers height (WH), body length (BL), body condition score (BCS), body weight (BWT), and average daily weight gain (ADG). Level of significance: ns not significant (P>0.05), * significant (P<0.05), ** highly significant (P<0.01), and *** very highly significant (P<0.001).

Gene expression pattern

To determine the expression pattern of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes a panel of tissues were collected from 20 genetically divergent Australian crossbred sheep. The genes were investigated directly for mRNA expression by qRT-PCR using the subcutaneous adipose and muscle tissues of sheep fed a diet supplemented with either 0, 10 or 20% *Spirulina*. The qRT-PCR results were calibrated and normalized using two housekeeping genes (*UBC* and *PPIA*) and the qBase relative quantification excel application (Pfaffl, 2001) for automated analysis.

Gene expression in subcutaneous adipose tissue

The relative mRNA expression levels of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes analysed in the SAT are presented in Figure 3.1. *ADRB3*, *BTG2*, and *FASN* showed higher expression levels in tissues from sheep that received 10 or 20% *Spirulina* supplement relative to 0% *Spirulina*. In contrast, *Spirulina* supplementation did not alter the mRNA expression of the *AANAT* gene in SAT.

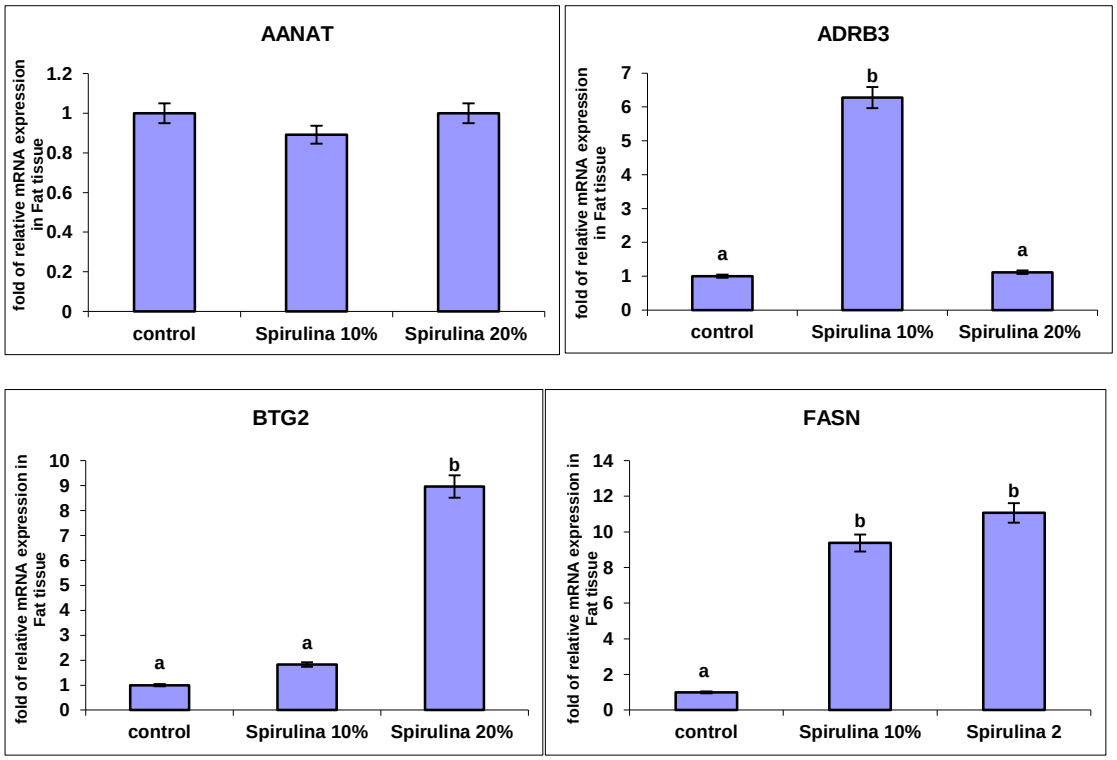

Figure 3.1 Relative expression levels of AANAT, ADRB3, BTG2 and FASN genes in the subcutaneous adipose tissue (SAT) of Australian crossbred sheep fed 0, 10% and 20% Spirulina supplementation. Each value was normalised to UBC and PPIA expression. Least square means with different superscripts differ by at least P<0.05

Ten percent dietary *Spirulina* supplementation up-regulated the mRNA expression of the *ADRB*3 gene (P<0.001), corresponding to a fold-change of 5.27 compared to the control treatment (Figure 3.1 and Table 3.2). *Spirulina* supplementation of 20% resulted in a down-regulation of *ADRB3* mRNA levels, which is a 5.08-fold lower than the 10% *Spirulina* condition and similar to the control condition level (i.e., 1.19-fold higher). The mRNA expression level of the *BTG*2 gene was significantly higher (*P*<0.001) (Table 3.2) in tissues from sheep supplemented with 20% *Spirulina* compared to either the 10% or control conditions. Ten percent *Spirulina* dietary supplement resulted in an up-regulation (0.83-fold) of *BTG*2 mRNA levels compared to the control, but this was not statistically significant. However, 20% *Spirulina* supplementation resulted in a significantly higher expression level (7.95-fold

increase) in SAT compared to controls. A similar expression profile was observed for the *FASN* gene as dietary *Spirulina* up-regulated the mRNA expression levels in both the 10 and 20% supplemented tissues; *FASN* expression increased 8.37-fold ($P<0.05$) under the 10% condition, and 10.06-fold under the 20% condition relative to controls (Table 3.2).

Gene expression in muscle

The relative mRNA expression levels of the four genes analysed in *longissimus dorsi* (*ls*) muscle tissues are presented in Figure 3.2.

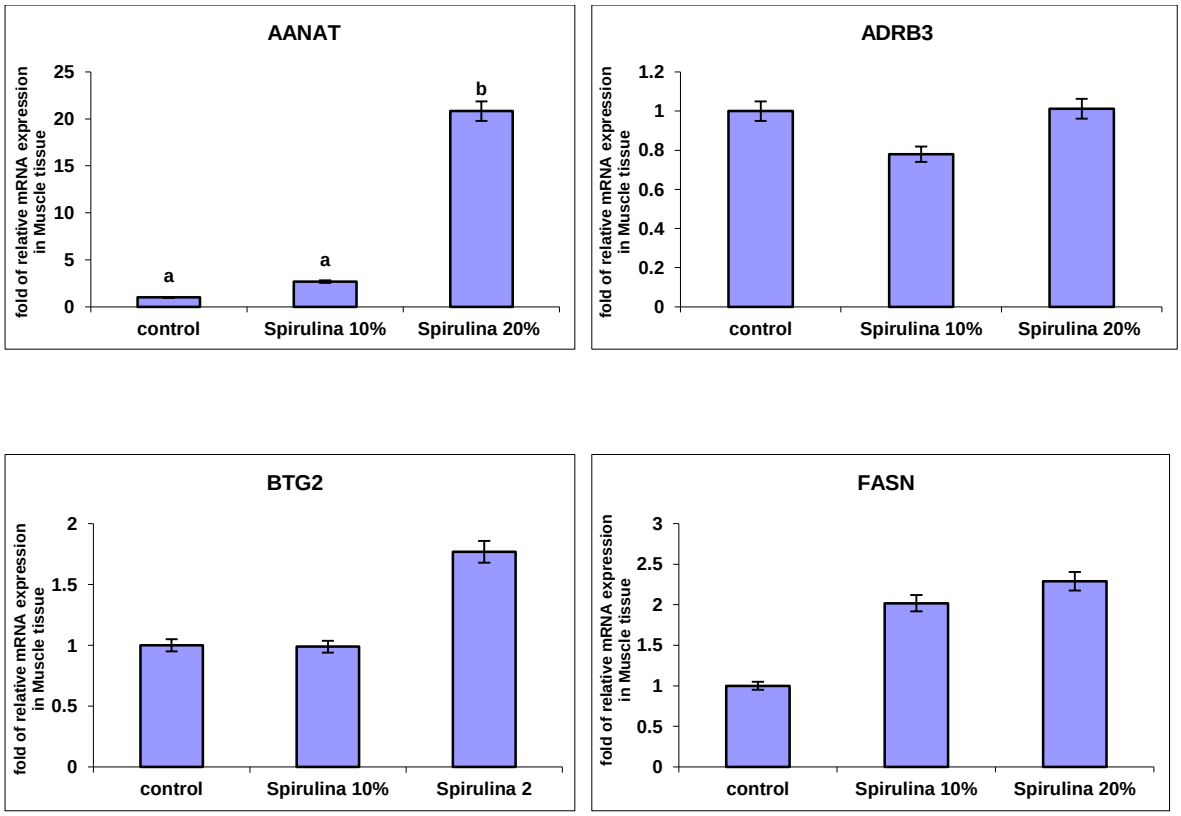

Figure 3.2 Relative expression levels of AANAT, ADRB3, BTG2, and FASN genes in the *longissimus dorsi* (*ls*) muscle of Australian crossbred sheep fed 0, 10**,** and 20% *Spirulina* supplementation. Each value was normalised to **the** *UBC* and *PPIA* expression**s**. Each value was normalised to *UBC* and *PPIA* expression**s**. Least square means with different superscripts differ by at least $P<0.05$.

It is evident that under both the 10 and 20% dietary *Spirulina* conditions, the mRNA expression levels of the *AANAT* gene increased in muscle tissues. However, this increase was only statistically significant in the 20% *Spirulina* condition, in which the *AANAT* mRNA expression level was 19.83-fold up-regulated compared to controls. In muscle tissue, supplementing with *Spirulina* had no significant effect ($P>0.05$) on the mRNA levels of the *ADRB3*, *BTG2*, and *FASN* genes (Table 3.2). However, under the 20% *Spirulina* supplementation, there was an up-regulation in the *BTG*2 (0.76-fold) and *FASN* (1.28-fold) mRNA levels, as compared to controls.

Discussion

Fats and fatty acid deposition in animal tissues has been attributed to a complex regulation network of lipogenic genes, genetics differences, and diet, although the molecular mechanisms underlying these systems remain to be characterized. Thus, understanding the factors affecting the depot-specific fat accretion and metabolism in sheep is extremely important. The present study addressed these aspects based on an experimental trial with four genetically diverse sheep breeds and three levels of protein-rich dietary supplement (*Spirulina*). The results reported here indicate that genetic background (i.e., breed), and to a lesser extent sex, determined the mRNA expression levels of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes. In order to elucidate the molecular mechanisms involved in this physiological process, these tissues and diet-specific variations are explained here through the transcript levels of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes.

Aralkylamine N-acetyltransferase

The Aralkylamine N-acetyltransferase gene encodes an acetyltransferase superfamily protein (Coon *et al.*, 1996). This acetyltransferase is the penultimate enzyme in melatonin synthesis

and controls the night/day rhythm in melatonin production in the vertebrate pineal gland. The Aralkylamine N-acetyltransferase protein catalyses the rate limiting step in the synthesis of melatonin from serotonin (Perez *et al.*, 2010). Melatonin is essential for the function of the circadian clock that influences activity and sleep (Coon et al., 1996, Coon et al., 1999). Aralkylamine N-acetyltransferase transcripts are found to be differentially expressed in high vs. low n-3 index (O3I) muscles, suggesting a role for melatonin in reducing oxidative damage, including that to PUFA (Holman et al., 2014, Perez et al., 2010).

The ability of melatonin to protect against lipid peroxidation has been repeatedly documented in many studies using animal and plant tissues (Holman *et al.*, 2014). Recently, Spanish scientists revealed that melatonin consumption assists in the control of weight gain since it stimulates the appearance of brown fat, a type of fat cell that burns calories instead of storing them (Jiménez-Aranda *et al.*, 2013). Their research demonstrated that melatonin treatment not only induced browning of inguinal white adipose tissue in Zucker diabetic fatty rats, but also increased the thermogenic activity of this tissue (Jiménez-Aranda *et al.*, 2013). Taken together, these findings highlight the anti-obesity effect of melatonin and explain its metabolic benefits of protection against oxidative degradation of PUFA in muscle tissue, which produce higher O3I levels (Jiménez-Aranda *et al.*, 2013).

Aralkylamine N-acetyltransferase transcription levels in muscle tissues are related to dietary *Spirulina* supplementation levels: 20% *Spirulina* supplementation resulted in a 7-fold and 20-fold up-regulation of *AANAT* mRNA levels relative to controls and 10% *Spirulina* treatments, respectively. These findings suggest that sheep receiving the 20% *Spirulina* supplement may have lost body weight due to the enhanced production of melatonin in their muscle tissue, as we observed; this is in accordance with phenotypic data from obese rats (Jiménez-Aranda *et al.*, 2013). Therefore, a high level of *Spirulina* supplementation may be involved with and

result in weight loss, which might occur through browning of the white adipose tissue and therefore increasing the n-3 levels in the fatty acid profile of the skeletal muscle tissue of sheep. However, further fatty acid analyses are required to examine this hypothesis.

Adrenergic beta-3 receptorAdrenergic beta-3 receptor encodes a protein belonging to the adrenergic receptor group of G-protein coupled receptors (Wu *et al.*, 2012). Adrenergic beta-3 receptor is located mainly in adipose tissue and plays a key role in regulating mammalian energy storage and expenditure under mediating effects from the sympathetic nervous system (Wu et al., 2011a, Hu et al., 2010). *ADRB3* is the principle mediator of the lipolytic and thermogenetic effect of high catecholamine (in particular, norepinephrine) concentration in brown and white adipose tissues in rodents (Wu et al., 2011a, Forrest et al., 2007b). The primary role of the receptor is proposed to be the regulation of the resting metabolic rate and lipolysis (Forrest *et al.*, 2003).

In a large number of studies, expression of the *ADRB3* gene was shown to be correlated with obesity in both humans and other mammals (Strosberg, 1997, Clément et al., 1995). Findings from various studies now provide a consistent picture of the important role of *ADRB3* in the regulation of lipid metabolism and make this protein an obvious target for drugs being designed to treat obesity (Strosberg, 1997).

It was observed that *ADRB3* transcription levels were significantly up-regulated in SAT under the medium (10%) dietary *Spirulina* condition, which is consistent with the observed phenotypic results with *ADRB3* that confirm weight gain in this group of lambs. The *ADRB3* mRNA expression levels under high (20%) *Spirulina* supplementation remained unchanged compared to the control group. One possible explanation for this observation is the negative

correlation between protein accretion and fat deposition rates that had been exacerbated by high feed protein levels (Holman *et al.*, 2012). In addition, excess protein may become delaminated and lost in the urine or become broken down in the liver, which could result in a condition of fatty liver and ketosis (Holman *et al.*, 2012). Thus, we are able to speculate that the medium level (10%) of *Spirulina* supplementation can be beneficial, giving higher production by fattening sheep. However, supplementing sheep with a higher dosage of *Spirulina* may result in lower efficiency in liver function and probably decrease the total production. Further research into *ADRB3* transcript levels in sheep liver would allow greater insight into the underlying mechanism in this species.

B-cell translocation gene 2

The mammalian *BTG/TOB* gene family belongs to the anti-proliferative (APRO) family, which regulate cell cycle progression in a variety of cell types (Kamaid and Giráldez, 2008, Mo et al., 2011). *B-cell translocation gene 2* is a prototypical member of the *BTG/TOB* family with anti-proliferative properties. The protein encoded by this gene controls cell cycle progression and proneural gene expression by acting as a transcription co-regulator that enhances or inhibits the activity of transcription factors (Kamaid and Giráldez, 2008, Mo et al., 2011).

Here we showed that *Spirulina* supplementation increased the *BTG2* transcription levels in SAT. However, only tissues from sheep receiving a high (20%) level of *Spirulina* supplement significantly over-expressed the *BTG2* gene. A number of studies have demonstrated that the *BTG2* gene has a potential role in muscle fibre size, intramuscular fat deposition, and weight loss (Kamaid and Giráldez, 2008, Mo et al., 2011). Here we suggest that the weight loss experienced by the high (20%) *Spirulina* supplementation group may be attributable to a decline of preadipocyte proliferation, an increase in energy expenditure, and a decline of energy uptake in adipocytes, which may be caused by an increase in *BTG2* expression.

Fatty acid synthase

Fatty acid synthase encodes a multifunctional enzyme that catalyses fatty acid synthesis (Berndt *et al.*, 2007). *FASN* is considered a fundamental enzyme in *de novo* lipogenesis in mammals, and its main function is to catalyse the synthesis of palmitate from acetyl-CoA and malonyl-CoA, in the presence of NADPH, into long-chain saturated fatty acids (LC-SFA) (Berndt et al., 2007, Holman, 2013). It has been shown that the *FASN* gene contributes to the regulation of body weight in humans and results in the development of obesity (Berndt et al., 2007, Boizard et al., 1998).

Both the medium (10%) and high (20%) level of *Spirulina* supplementation increased the transcription levels of *FASN* in both SAT and muscle tissues, which may have assisted these sheep in gaining weight.

The results demonstrate that supplementing sheep with 10% *Spirulina* can increase lamb production by increasing the transcription level of *ADRB3* and *FASN* genes in SAT. Also, this supplementation might also beneficially alter the fatty acid profile by reducing the oxidation of PUFA in skeletal muscle.

Conclusions

The results presented herein suggest that the mRNA levels of the *AANAT, ADRB3, BTG2*, and *FASN* genes in the SAT and muscle tissues are mainly influenced by dietary *Spirulina* level, whereas the effects of breed and sex, and their combined effects with diet, are not associated with the mRNA expression of the above genes. Taken together, our results show that lipid metabolism in SAT is more sensitive to diet than in muscle. These findings provide evidence

to support an intermediate level of dietary protein supplementation for achieving an optimal increase in the n-3 and n-6 content of red meat among Australian crossbred sheep.

Authors' contributions

This work was carried out in collaboration between all authors. Author AK participated in the feeding trial, ran the laboratory analyses, and wrote the first draft of the manuscript as part of his PhD project. Both AK and BWBH carried out the feeding trial, collected blood samples, and performed feed chemical analysis. Author AEOMA and PDN conceived the research idea, wrote the funding grant, experimental design, and made needed changes to the draft and final manuscript. All authors read and approved the final manuscript.

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Chapter 4

Effect of dietary supplementation of purebred and crossbred Australian lambs with *Spirulina* (*Arthrospira platensis*) *on* the mRNA expression and transcriptional analysis of Aralkylamine N-acetyltransferase, Adrenergic beta-3 receptor, B-cell translocation gene and Fatty Acid Synthase genes in the heart, kidney and liver

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Abstract

The demand for healthy, lean, and consistent prime lamb and organ products with low saturated and high polyunsaturated fatty acid (PUFA) contents, especially long-chain n-3 PUFA, has recently increased. Fat deposition in ruminant livestock can be altered by manipulating the genetics of the animal and its dietary supplementation regime. The aim of this study was to assess the effect of dietary Spirulina supplementation on the messenger RNA expression profiles of genes controlling fatty acid metabolism in the heart, kidney, and liver of Australian purebred and crossbred prime lambs. Fourty-eight weaned lambs belonging to four breeds under the same management conditions were maintained on ryegrass pasture and randomly allocated to three treatment groups, including the control group (unsupplemented or 0%) and two levels of Spirulina supplementation: Low (100mL/head/day of 1g Spirulina powder dissolved in 10 mL of water [10%wt:vol]) and High (200mL/head/day of 2g of Spirulina dissolved in 10 mL of water [20%wt:vol]). All lambs had ad libitum access to water and were supplemented for 9 weeks after 3 weeks of adjustment before they were slaughtered. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used after slaughter to assess the transcription levels of Aralkylamine N-acetyltransferase (AANAT), Adrenergic beta-3 receptor (ADRB3), B-cell translocation gene 2 (BTG2), and Fatty acid synthase (FASN) genes in 432 heart, kidney, and liver samples. The fatty acid (FA) compositions of these organs were analysed by mass spectrophotometry and gas chromatography. Both the low and high levels of Spirulina supplementation regimes strongly up-regulated the transcription of all the selected genes and increased n-3 and n-6 PUFA compositions in the tested organs. Although sheep breed and sex did not influence the gene expression patterns in these tissues, the significant variations in organ fatty acid composition in response to Spirulina supplementation underpin the genetics-nutrition interactions that could be of practical importance for manipulating meat quality in the Australian dual-purpose prime lamb industry.

Keywords: *Spirulina*, Sheep, AANAT, ADRB3, BTG2, FASN, gene expression, fatty acids

Introduction

Several internal and external factors influence the quantity and quality of lipids in animal products. Dietary manipulation is a principal method for altering the fatty acid (FA) content and composition of livestock tissues and organs (Wood and Enser, 1997). *Spirulina* (*Arthrospira platensis*) is a blue-green cyanobacterial microalga that contains 60-70% protein, high levels of carotenoids, essential vitamins, minerals, and fatty acids (Ciferri, 1983, Holman and Malau-Aduli, 2013). *Spirulina* has been trialled as a supplementary feed in many animal species, and its inclusion in the diet of sheep has proven to be an effective nutritional strategy for increasing sheep meat production (Holman et al., 2012, Holman and Malau-Aduli, 2013). Both the genetic background of the animal and dietary supplementation can alter fat deposition and its FA composition in sheep (Kouba and Mourot, 2011). However, the molecular mechanisms underlying fat deposition and FA composition in sheep are not yet fully understood. To our knowledge, there is no available information on the molecular genetics and genomics-nutrition interactions between ovine organs and dietary supplementation with *Spirulina* and the potential for polyunsaturated fatty acid (PUFA) enhancement in dual-purpose sheep. Therefore, the aim of this study was to investigate changes in the mRNA expression and transcriptional patterns of the following genes controlling lipid metabolism in the heart, kidney, and liver of sheep under various *Spirulina* supplementation regimes: Aralkylamine N-acetyltransferase (AANAT), adrenergic beta-3 receptor (ADRB3), B-cell translocation gene 2 (BTG2), and fatty acid synthase (FASN).

Materials and methods

Animal management, experimental design and data collection

All procedures involving animals were approved by the University of Tasmania Animal Ethics Committee and were conducted in accordance with the 1993 Tasmanian Animal Welfare Act and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The experimental flock at the University of Tasmania Farm, Cambridge, Hobart, utilised for this study

comprised forty-eight weaned lambs from purebred Merino dams sired by White Suffolk, Black Suffolk, Dorset, and Merino rams under the same management conditions. All animals were maintained on ryegrass pastures as the basal diet. At six weeks of age, they were balanced by sire breed and gender and randomly allocated into three treatment groups: the *control* group grazing without *Spirulina* (0%), *low* (100mL/head/day in the ratio of 1g of *Spirulina* powder:10mL of water or 10% wt/vol), and *high* (200mL/head/day in the ratio of 2g of *Spirulina* powder:10mL of water or 20% wt/vol) *Spirulina* supplementation levels. The *Spirulina* powder was purchased from a commercial producer in Darwin, Northern Territory, Australia (TAAU, NT, Aus). Lambs were daily supplemented according to their assigned *Spirulina* treatment group before being released into paddocks for grazing. The supplementary feeding trial continued for nine weeks after an initial three weeks of adjustment. Liveweight (LWT), body condition score (BCS), body length (BL), withers height (WH), chest girth (CG), and average daily gain (ADG) were recorded weekly over this period. Details of the procedures for recording these growth and body conformation measurements had previously been published (Holman *et al.* 2014). At the end of the feeding trial, blood samples were taken by jugular venipuncture before the animals were slaughtered at a commercial abattoir in Gretna, Tasmania, for carcass and sensory evaluation of meat quality. Heart, kidney, and liver tissue samples were immediately removed from each carcass, frozen in liquid nitrogen, and transported to the laboratory, where they were stored at -80 °C until RNA extraction and further analyses.

RNA extraction and cDNA synthesis

Total RNA was isolated from a total of 432 frozen tissue samples using a TRIzol® Plus RNA Purification Kit (Life Technologies Pty Ltd. Victoria, Australia). Homogenisation of the tissue samples in TRIzol® Reagent was performed using a tissue lyser (Qiagen Ltd., Crawley, UK), and the RNA was subsequently extracted using chloroform and precipitated using isopropanol. Quantity and quality of total RNA was assessed using the NanoDrop 8000 spectrophotometer (NanoDrop, Wilmington, DE). RNA quality was verified by ensuring all RNA samples had an absorbance ratio (A260/280) between 1.8 and 2. RNA

samples were treated with PureLink^{TM}DNase (Life Technologies Pty Ltd. Victoria, Australia) and purified using the RNeasy Mini Kit (Qiagen Ltd.). DNase-treated and purified total RNA was then reverse transcribed to cDNA using mixed oligo dT/random hexamer primers and the Tetro cDNA Synthesis Kit (Bioline Pty Ltd. NSW, Australia) according to the manufacturer's instructions and stored at -80 °C for subsequent analyses.

Primer design and reference gene selection

All candidate and reference gene primers in this study (Table 4.1) were designed using the Primer3 web-based software program (http://frodo.wi.mit.edu/primer3/) and obtained from a commercial supplier (GeneWorks Pty Ltd., SA, Australia).

Table 4.1 Quantitative real-time PCR (qRT-PCR) oligonucleotide primers

[a]Gene symbol	qPCR Primers		[b]T_a	Amplicon Size (bp)
	Forward Primer	Reverse Primer		
AANAT	ACTGACCTTCACGGAGATGC	TTCACTCATTCTCCCCGTTC	60	211
ADRB3	TCAGTAGGAAGCGGGTCGGG	GGCTGGGGAAGGGCAGAGTT	60	291
BTG2	CTGGAGGAGAACTGGCTGTC	AAAACAATGCCCAAGGTCTG	60	194
FASN	GTGTGGTACAGCCCCTCAAG	ACGCACCTGAATGACCACTT	60	110
Reference genes				
UBC	CGTCTTAGGGGTGGCTGTTA	AAATTGGGGTAAATGGCTAGA	60	90
PPIA	TCATTTGCACTGCCAAGACTG	TCATGCCCTCTTTCACTTTGC	60	72

[a]Aralkylamine N-acetyltransferase=AANAT, β3-adrenergic receptor=ADRB3, B-cell translocation gene 2=BTG2, Fatty acid synthase=FASN, Ubiquitin C=UBC, Peptidyl-prolyl cis-trans-isomeraseA=PPIA, [b]T_a=Empirical annealing Temperature.

Primer specificity was checked using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). All primers were validated using pooled cDNA samples. A standard curve was generated using serial dilutions of pooled cDNA. PCR products generated by amplification were sequenced to verify their primer specific

identity (Beckman Coulter CEQ^{TM} 8000 Series Genetic Analysis System, University Tasmania). To determine the relative gene expression levels, suitable highly stable reference genes were required. In the current study of five tested reference genes, *Ubiquitin C* (*UBC*) and *Peptidyl-prolyl cis-trans isomeraseA* (*PPIA*) were the most stable; hence, they were used to normalise the gene expression data. The principle behind the selection of the reference genes was that the expression ratio of two ideal reference genes should be constant across all samples. The expression stability of the reference genes was validated using geNorm (Version 3.5) by calculating the gene expression stability measure (M value).

Quantitative real time PCR (qRT-PCR)

Following reverse transcription, cDNA quantity was determined and standardised to the required concentration for qRT-PCR. Triplicate 20 µL reactions were carried out in a 72-well Rotor-Gene (QIAGEN GmbH, Hilden, Germany) system, containing 4 µL cDNA (50 ng), 10 µL 2×SensiFAST SYBR No-ROX Mix (Bioline Pty Ltd., NSW, Australia), 4.4 µL DEPC H_2O, and 0.8 µL forward and reverse primers (100 fmol). Assays were performed using a Rotor-Gene 3000 (QIAGEN Pty Ltd., VIC, Australia) with the following cycling parameters: 95 °C for 2 min (polymerase activation); 40 cycles of 95 °C for 5 s (denaturation), 60 °C for 10 s (annealing), and 72 °C for 5 s (extension). Gene expression levels were recorded as Ct values (i.e., the number of PCR cycles at which the fluorescence signal is detected above the threshold value), and all samples were run in triplicate. Amplification efficiencies were determined for all candidate and reference genes using the formula E=10^(−1/slope), with the slope of the linear curve of cycle threshold (Ct) values plotted against the log dilution (Higuchi *et al.*, 1993). Primer concentrations were optimised for each gene, and disassociation curves were examined for the presence of a single PCR product. The efficiency of the reaction was calculated using a 5-fold serial dilution of cDNA and generation of a standard curve. All PCR efficiency coefficients were between 1.7 and 1.8, and therefore they were deemed acceptable. The software package Rotor-Gene 3000 version 6.0.16 (Qiagen Pty Ltd., VIC, Australia) was used for efficiency correction of the raw Ct values, inter-

plate calibration, averaging of replicates, normalisation to the reference gene, calculation of quantities relative to the highest Ct, and log2 transformation of the expression values for all genes.

Gene expression pattern

To determine the expression pattern of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes, 432 heart, kidney, and liver tissue samples were collected from 48 genetically divergent Australian purebred and crossbred prime lambs supplemented with either no, low, or high levels of *Spirulina*. The genes were investigated for mRNA expression by qRT-PCR. The qRT-PCR results were calibrated and normalized using two housekeeping genes (*UBC* and *PPIA*), and the qBase relative quantification excel application (Pfaffl, 2001) was utilized for automated analysis.

Lipid extraction and fatty acid analysis

All tissue samples were extracted using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). This involved a single-phase overnight extraction using $CHCl_3$: MeOH:H2O (1:2:0.8 v/v), followed by phase separation with the addition of $CHCl_3$:saline H_2O (1:1 v/v). Total lipid extract was obtained by rotary evaporation of the lower chloroform phase. From each sample, an aliquot of extracted total lipid was transmethylated in MeOH:$CHCl_3$:HCl (10:1:1 [v/v]) for 2 h at 80 °C. Milli-Q H_2O (1 ml) was then added before FA methyl esters (FAME) were extracted with hexane: chloroform (4:1 [v/v]). Then it was reduced under a nitrogen stream, and a known concentration of an internal injection standard (19:0 FAME) was added. An Agilent Technologies 7890B gas chromatograph (GC) (Palo Alto, California USA) equipped with an Equity™-1 fused silica capillary column (15 m × 0.1 mm internal diameter and 0.1 µm film thickness), a flame ionisation detector, a split/splitless injector, and an Agilent Technologies 7683 B Series autosampler was used in the analysis. Samples were injected in splitless mode, carried by helium gas, at an oven temperature of 120 °C. Post-injection, the oven temperature was raised to 270 °C at 10 °C/min, then to 310 °C at 5 °C/min. Peaks were quantified by the Agilent Technologies ChemStation software (Palo Alto, California USA). FA identities were confirmed using GC-mass

spectrometric (GC/MS) analysis. These were performed using a Finnigan Thermoquest GCQ GC-MS fitted with an on-column injector and using the Thermoquest Xcalibur software (Austin, Texas USA). The GC had a HP-5 cross-linked methyl silicone-fused silica capillary column (50 m × 0.32 mm internal diameter). The carrier gas used was helium, with operating conditions previously described (Miller *et al.*, 2006).

Statistical Analysis

Individual FAs and the summations of saturated, monounsaturated, and PUFAs were computed as percentages of total FAs. Summary statistics were computed with means, standard deviations, and minimum and maximum values to check for errors and outliers. A generalised linear model (GLM) in SAS (2009) was used in computing the fixed effects of *Spirulina* supplementation level, tissue, sire breed and sex, and their interactions on the mRNA expression level of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes in the heart, kidney, and liver tissues. Bonferroni's probability pairwise comparison test was used to separate mean differences, with the level of significance defined as $P<0.05$.

Results

Gene expression in the heart

The relative mRNA expression levels of *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes in the heart are presented in Figure 4.1.

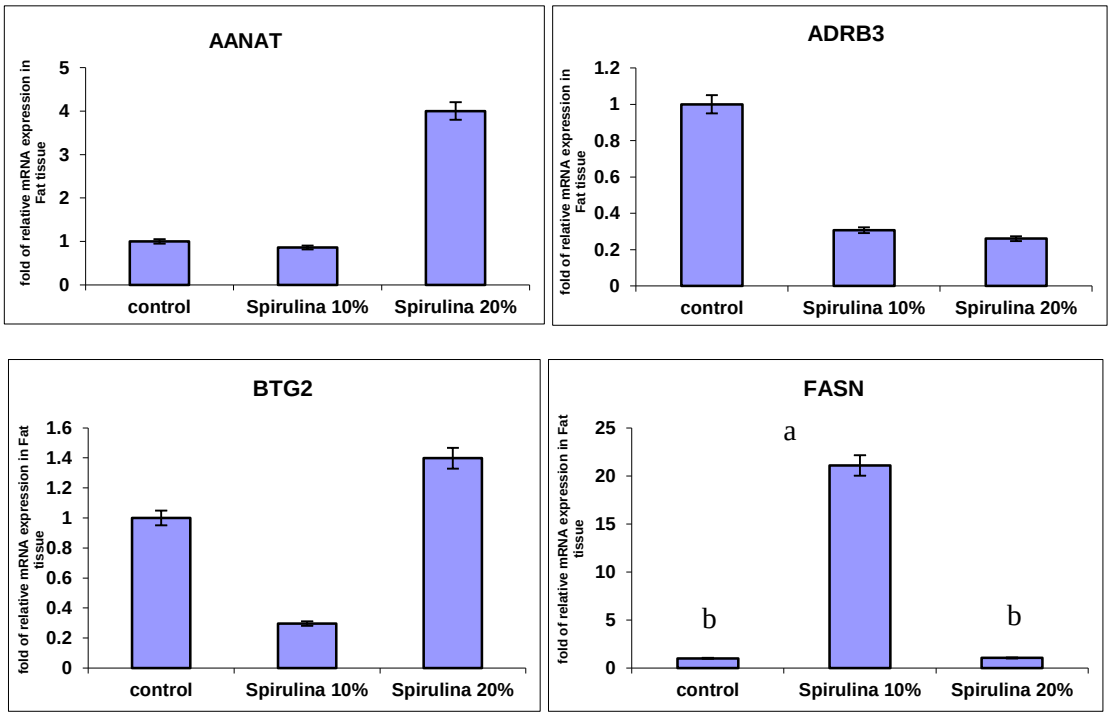

Figure 4.1 Relative gene expressions in the heart tissue of Australian prime lambs in the control **(0%)**, low (10%)**,** and high (20%) levels of *Spirulina* supplementation. Each value was normalised to *UBC* and *PPIA* expressions. Least square means with different superscripts differ by at $P<0.05$

The *FASN* expression was higher in the tissues of prime lambs that received 10% *Spirulina* supplementation as compared to those that received 0 or 20% *Spirulina*. Ten percent dietary *Spirulina* supplementation significantly up-regulated mRNA expression of the *FASN* gene ($P<0.041$), corresponding to a 21-fold change as compared to the control (0%) and high supplementation (20%) levels. In contrast, 20% *Spirulina* supplementation did not alter the mRNA expression of the *ADRB3* gene in the heart. Similarly, 10% *Spirulina* supplementation did not alter the mRNA expression of *AANAT* gene in the heart compared to 0% supplementation. However, under the 20% supplementation regime, the *AANAT* transcript was higher than in the control group. Both 10% and 20% *Spirulina* supplementation regimes resulted in seemingly reduced *ADRB3* transcription levels in the heart. *BTG2* transcription levels were marginally down-regulated and up-regulated in the heart of 10% and 20% of *Spirulina* supplemented lambs, respectively.

Gene expression in the kidney

The relative mRNA expression levels of the four genes in the kidney are presented in Figure 5.2.

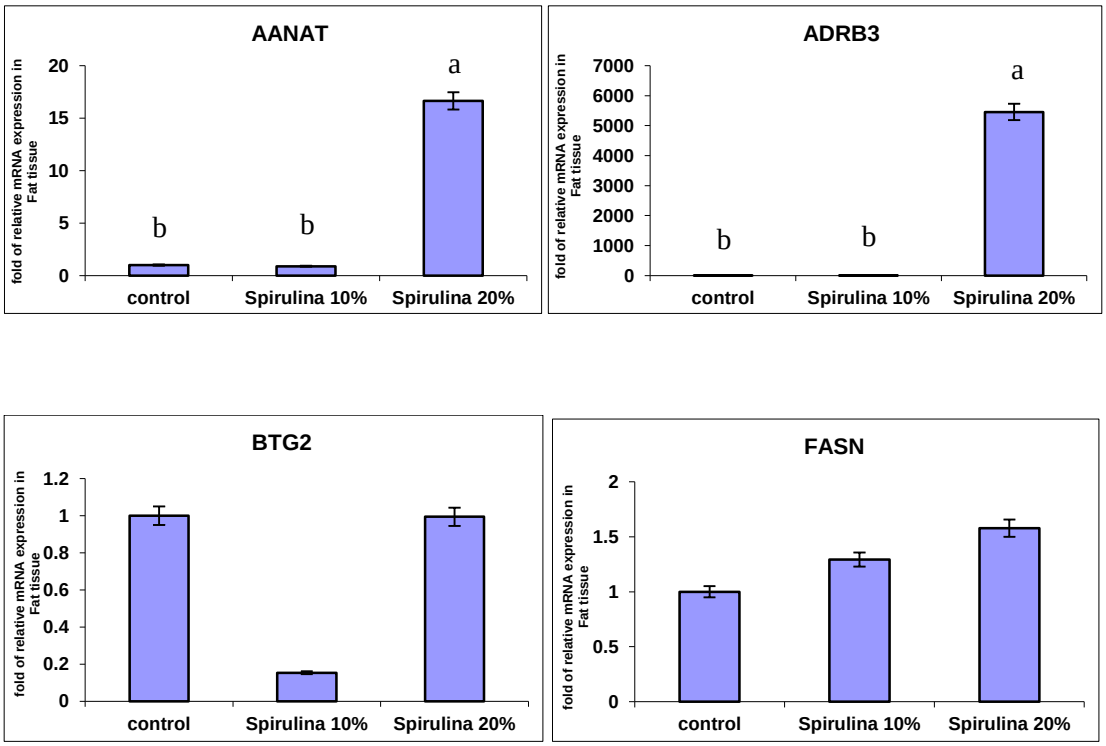

Figure 4.2 Relative gene expressions in the kidney of Australian prime lambs in the control (0%), low (10%), and high (20%) levels of *Spirulina* supplementation treatment groups. Each value was normalised to *UBC* and *PPIA* expressions. Least square means with different superscripts differ at $P<0.05$

In the 20% dietary *Spirulina* supplementation group of lambs, the mRNA expression levels of *AANAT* and *ADRB3* genes increased by 16.64-fold and 5453-fold, respectively, compared to the control group (0% *Spirulina*). Supplementing the lambs with *Spirulina* had no significant effect ($P>0.05$) on the mRNA levels of the *BTG2* and *FASN* genes (Figure 4.2). However, kidneys from lambs in the 10% *Spirulina* supplementation group demonstrated a down-regulation in the *BTG*2 transcript (0.15-fold), compared to the control group.

Gene expression in the liver

The relative mRNA expression levels of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes in the liver are presented in Figure 4.3.

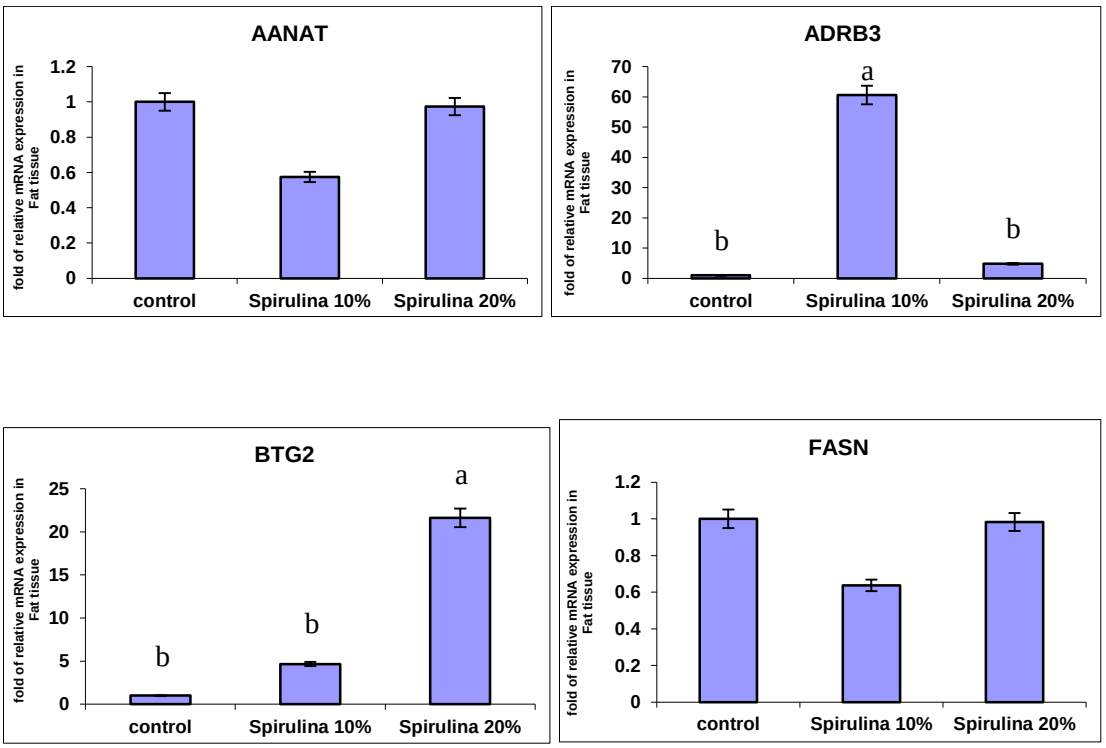

Figure 4.3 Relative gene expressions in the liver of Australian prime lambs in the control (0%), low (10%), and high (20%) levels of Spirulina supplementation treatment groups. Each value was normalised to UBC and PPIA expressions. Least square means with different superscripts differ at P<0.05.

Higher *ADRB3* and *BTG2* mRNA expression levels were detected in the liver of lambs supplemented with 10 or 20% *Spirulina* than their control counterparts in the 0% *Spirulina* group. In contrast, *Spirulina* supplementation did not alter the mRNA expression levels of the *AANAT* and *FASN* genes in the liver. Ten percent dietary *Spirulina* supplementation significantly up-regulated mRNA expression of the *ADRB*3 gene (P=0.032), corresponding to a 60.59-fold increase compared to the control treatment (Figure 4.3). Twenty percent dietary *Spirulina* supplementation significantly up-regulated the mRNA expression of the *BTG*2 gene (P=0.024), corresponding to a 21.63-fold change compared to the control treatment (Figure 4.3). In liver tissue, supplementing with *Spirulina* had no significant effect (P>0.05) on the mRNA levels of the *AANAT* and *FASN* genes (Figure 4.3).

Table 4.2 Mean fatty acid compositions (% of total fatty acids) of *Spirulina* and standard error of mean (SEM)

Fatty acid	Mean (% total FA)	SEM
16:0 Palmitic acid	24.8	1.4
16:1n-9c Palmitoleic acid	3.7	0.5
17:0 Heptadecanoic acid, or margaric acid	1.7	0.1
18:0	6.3	0.9
18:1n-9 Oleic acid	9.8	1.1
18:2n-6 Linoleic acid	12.2	1.4
18:3n-3 α-Linolenic acid	4.46	0.3
20:0 Arachidic acid	2.1	0.2
20:2n-6 Eicosadienoic acid	1.9	0.4
20:3n-6 Dihomo-γ-linolenic acid (DGLA)	2.2	0.2
20:5n-3 Eicosapentaenoic acid	1.95	0.1

Table 4.3. Heart fatty acid composition (% total fatty acids), standard error of mean (SEM), number of lambs and samples (*n*) [a,b]

	Spirulina supplementation treatment groups							
Fatty acids	Control (n=12)		Low (n=12)		Medium (n=12)		High (n=12)	
	108 samples		108 samples		108 samples		108 samples	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	0.7	0.2^{B}	1.2	0.4^{A}	0.8	0.2^{BA}	0.5	0.1^{B}
15:0	0.4	0.0^{A}	0.2	0.0^{B}	0.0	0.0^{B}	0.4	0.0^{A}
16:1n-9c	0.2	0.0	0.2	0.0	0.3	0.0	0.2	0.0
16:1n-7c	0.4	0.1	0.1	0.1	0.4	0.1	0.3	0.1
16:0	15.1	0.1^{A}	14.7	0.6^{AB}	14.1	0.1^{B}	15.0	0.4^{A}
17:0	1.3	0.0	1.5	0.1	1.3	0.0	1.4	0.0
18:2n-6	17.1	1.2	15.5	1.6	16.5	1.5	15.7	1.4
18:3n-3	3.6	0.4	2.1	0.1	3.4	0.3	3.0	0.2
18:1n-9	18.1	0.4^{B}	19.9	0.2^{A}	19.8	0.1^{A}	19.0	0.1^{AB}
18:1n-7c	1.9	0.1	2.0	0.2	2.0	0.1	1.9	0.1
18:1n-7t	2.1	0.2	1.7	0.2	2.3	0.2	2.2	0.2
18:0	19.3	1.0	21.0	1.8	21.5	1.4	21.6	1.3
20:4n-6	5.6	0.5	4.6	0.8	4.1	0.6	4.8	0.7
20:5n-3	3.1	0.6	1.5	0.2	2.3	0.2	2.6	0.6
20:3n-6	0.6	0.0	0.5	0.1	0.5	0.1	0.6	0.0

20:4n-3	0.1	0.0^{B}	0.2	0.0^{A}	0.1	0.0^{B}	0.1	0.0^{B}
20:2n-6	0.0	0.0^{B}	0.0	0.0^{B}	0.1	0.0^{A}	0.0	0.0^{B}
20:0	0.1	0.0^{C}	0.3	0.0^{A}	0.1	0.0^{C}	0.2	0.0^{B}
22:5n-6	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
22:6n-3	1.3	0.2	0.9	0.2	0.7	0.1	1.0	0.2
22:5n-3	1.9	0.3	1.6	0.3	1.2	0.2	1.5	0.2
22:0	0.3	0.1	0.2	0.0	0.1	0.0	0.3	0.1
23:0	0.2	0.1	0.3	0.0	0.1	0.0	0.3	0.0
24:0	0.2	0.0	0.2	0.0	0.1	0.0	0.2	0.0
∑SFA	37.8	1.2	41.1	3.3	40.1	1.8	40.7	1.5
∑MUFA	28.2	0.9	30.9	1.4	30.3	0.9	29.2	1.1
∑PUFA	34.0	1.4	27.9	3.3	29.6	2.5	30.1	2.0
∑n-3	10.0	1.0	6.3	0.8	7.8	0.6	8.3	0.9
∑n-6	23.4	1.3	21.0	2.5	21.4	2.0	21.4	1.7

[1] Means with different superscripts $^{A, B, C, D, E}$ within rows significantly differ (P<0.05).

[2] ∑SFA is the sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0; ∑MUFA is the sum of 14:1n-5, 15:1n-6, 16:1n-9, 16:1n-7, Br17:1, 17:1n-8+a17:0, 17:1, 18:1n-9, 18:1n-7, 18:1n-5, 18:1, 19:1, 20:1n-11, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9, 22:1n-11, 22:1n-7, 24:1n-11, 24:1n-9, 24:1n-7; ∑PUFA is the sum of 16:3+16:4, 16:2, 18:4n-3, 18:3n-6, 18:2n-6, 18:3n-3, 20:4n-3, 20:4n-6, 20:5n-3, 20:3n-6, 20:2n-6, 21:5n-3, 22:6n-3, 22:5n-3, 22:5n-6, 22:4n-6, 24:6n-3, 24:5n-3; ∑n-3 PUFA is the sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 21:5n-3, 22:6n-3, 22:5n-3, 24:6n-3, 24:5n-3; ∑n-6 PUFA is the sum of 18:2n-6, 18:3n-6, 20:4n-6, 20:3n-6, 20:2n-6, 22:5n-6, 22:4n-6.

Fatty acid compositions in the heart, kidney and liver

The fatty acid composition of *Spirulina* is depicted in Table 4.2. In the heart (Table 4.3), the 15:0 FA composition was lowest (0.2%) in the heart of lambs supplemented at 10% *Spirulina* levels. Palmitic acid (16:0) composition differed (P<0.05) in heart tissues of lambs supplemented at 10% *Spirulina* level compared to the control group. Heart tissues of lambs supplemented at the 10% level had the highest 20:2n-6 composition (0.1%), which was significantly different (P<0.01) to the other supplementation groups. It was also clear that the percentage of 18:1n-9 was higher in both 10% and 20% supplementation levels compared to the control group (Table 4.3).

Table 4.4. Kidney fatty acid composition (% total fatty acids), standard error of mean (SEM), number of lambs and samples (*n*) [a,b]

	Spirulina supplementation treatment group							
Fatty acids	Control (n=12)		Low (n=12)		Medium (n=12)		High (n=12)	
	108 samples		108 samples		108 samples		108 samples	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	0.2	0.1	0.2	0.2	0.1	0.1	0.2	0.1
15:0	0.3	0.0	0.2	0.1	0.3	0.1	0.3	0.0
16:1n-9c	0.2	0.0	0.1	0.1	0.2	0.0	0.2	0.1
16:1n-7c	0.3	0.0	0.3	0.1	0.4	0.1	0.3	0.1
16:0	19.2	0.8	19.0	1.3	19.3	1.7	18.2	0.3
17:0	1.4	0.1	1.6	0.1	1.3	0.1	1.3	0.1
18:2n-6	8.9	0.4	10.7	0.6	9.0	0.6	9.7	0.4
18:3n-3	1.9	0.3	1.6	0.4	4.4	2.3	2.1	0.3
18:1n-9	17.1	0.3^{A}	15.8	0.4^{B}	15.2	0.2^{B}	15.2	1.0^{B}
18:1n-7c	1.4	0.1	1.8	0.1	1.5	0.2	1.4	0.1
18:1n-7t	1.6	0.2	0.9	0.1	1.3	0.3	1.2	0.2
18:0	22.5	1.0	21.1	1.1	19.0	1.6	19.8	1.2
20:4n-6	7.9	1.0	9.4	1.5	8.4	1.3	10.3	1.3
20:5n-3	5.0	0.9	2.7	0.7	5.1	0.9	5.5	0.7
20:3n-6	0.6	0.1	0.7	0.1	0.5	0.1	0.7	0.1
20:4n-3	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
20:2n-6	0.1	0.0	0.1	0.0	0.1	0.0	0.0	0.0
20:0	0.2	0.0	0.1	0.0	0.2	0.0	0.2	0.0
22:5n-6	0.1	0.1^{B}	0.0	0.1^{B}	0.9	0.0^{A}	0.5	0.1^{A}
22:6n-3	2.5^{B}	0.2	2.7	0.1^{B}	2.8	0.2^{A}	3.4	0.1^{A}
22:5n-3	2.7	0.3	2.6	0.3	2.6	0.4	3.2	0.2
22:0	1.0	0.1	0.9	0.2	1.5	0.3	1.0	0.1
23:0	0.2	0.1	0.2	0.1	0.3	0.1	0.2	0.1
24:0	0.8	0.1	0.9	0.3	1.3	0.4	0.7	0.1
∑SFA	46.6	1.6	44.7	2.3	44.4	0.9	42.7	1.3
∑MUFA	22.7	0.7	23.5	1.8	22.6	1.6	21.9	1.0

∑PUFA	30.8	0.5[B]	31.8	0.1[B]	33.9	0.5[A]	35.4	0.6[A]
∑n-3	12.5	1.5	9.8	0.7	14.7	1.8	14.0	0.9
∑n-6	18.0	1.2	21.8	1.2	18.1	1.6	21.0	1.5

[a] Means with different superscripts [A, B, C, D, E] within rows significantly differ ($P<0.05$).

[b] ∑SFA is the sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0; ∑MUFA is the sum of 14:1n-5, 15:1n-6, 16:1n-9, 16:1n-7, Br17:1, 17:1n-8+a17:0, 17:1, 18:1n-9, 18:1n-7, 18:1n-5, 18:1, 19:1, 20:1n-11, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9, 22:1n-11, 22:1n-7, 24:1n-11, 24:1n-9, 24:1n-7; ∑PUFA is the sum of 16:3+16:4, 16:2, 18:4n-3, 18:3n-6, 18:2n-6, 18:3n-3, 20:4n-3, 20:4n-6, 20:5n-3, 20:3n-6, 20:2n-6, 21:5n-3, 22:6n-3, 22:5n-3, 22:5n-6, 22:4ω6, 24:6n-3, 24:5n-3; ∑n-3 PUFA is the sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 21:5n-3, 22:6n-3, 22:5n-3, 24:6n-3, 24:5n-3; ∑n-6 PUFA is the sum of 18:2n-6, 18:3n-6, 20:4n-6, 20:3n-6, 20:2n-6, 22:5n-6, 22:4n-6.

Table 4.5 Liver fatty acid composition (% total fatty acids), standard error of mean (SEM), number of lambs and samples[a,b,c]

	Spirulina supplementation treatment group							
Fatty acids	Control (n=12) 108 samples		Low (n=12) 108 samples		Medium (n=12) 108 samples		High (n=12) 108 samples	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	0.6	0.2[A]	0.5	0.2[A]	0.3	0.1[B]	0.3	0.1[B]
15:0	0.5	0.1	0.5	0.1	0.5	0.0	0.4	0.0
16:1n-9c	0.4	0.1	0.4	0.1	0.4	0.0	0.4	0.0
16:1n-7c	0.6	0.1	0.4	0.1	0.7	0.1	0.5	0.1
16:0	19.8	1.4	22.0	1.0	18.9	0.9	19.7	1.2
17:0	1.5	0.1	2.0	0.1	1.3	0.1	1.5	0.1
18:2n-6	6.4	0.5	6.2	0.7	6.4	0.4	6.4	0.5
18:3n-3	3.0	0.2	2.4	0.7	3.4	0.3	3.0	0.3
18:1n-9	21.0	1.1	25.6	1.1	22.0	1.0[B]	22.1	1.0[B]
18:1n-7c	1.3	0.1	1.3	0.1	1.3	0.0	1.2	0.0
18:1n-7t	2.4	0.4	2.0	0.1	1.8	0.3	2.4	0.3
18:0	22.5	2.6	23.9	1.8	21.9	1.4	23.3	1.3
20:4n-6	4.4	0.9	2.9	0.8	4.4	0.6	3.8	0.7
20:5n-3	1.0	0.6[B]	3.1	0.4[A]	3.5	0.5[A]	2.8	0.6[A]
20:3n-6	0.3	0.1[B]	0.5	0.1[A]	0.6	0.1[A]	0.5	0.1[A]

20:0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
22:5n-6	0.1	0.0[B]	0.1	0.9[B]	1.1	0.0[A]	0.1	0.0[B]
22:6n-3	3.9	0.7	1.9	0.3	3.7	0.3	3.0	0.5
22:5n-3	3.3	0.5	1.6	0.4	3.8	0.4	3.3	0.5
22:0	0.2	0.1	0.1	0.0	0.2	0.1	0.2	0.1
23:0	0.1	0.1	0.2	0.1	0.2	0.1	0.2	0.1
24:0	0.2	0.1	0.1	0.0	0.2	0.1	0.2	0.1
∑SFA	46.1	2.2	50.0	2.5	44.4	1.2	46.7	1.7
∑MUFA	28.6	1.3	32.4	1.3	29.0	1.1	29.8	1.1
∑PUFA	25.3	2.7	17.6	2.4	26.6	1.6	23.5	2.3
∑n-3	13.4	1.7	6.9	1.5	14.6	1.1[A]	12.3	1.5
∑n-6	11.6	1.3	10.5	1.0	11.7	1.0	11.0	1.2

[a] Means with different superscripts [A, B, C, D, E] within rows significantly differ ($P<0.05$).

[b] ∑SFA is the sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0; ∑MUFA is the sum of 14:1n-5, 15:1n-6, 16:1n-9, 16:1n-7, Br17:1, 17:1n-8+a17:0, 17:1, 18:1n-9, 18:1n-7, 18:1n-5, 18:1, 19:1, 20:1n-11, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9, 22:1n-11, 22:1n-7, 24:1n-11, 24:1n-9, 24:1n-7; ∑PUFA is the sum of 16:3+16:4, 16:2, 18:4n-3, 18:3n-6, 18:2n-6, 18:3n-3, 20:4n-3, 20:4n-6, 20:5n-3, 20:3n-6, 20:2n-6, 21:5n-3, 22:6n-3, 22:5n-3, 22:5n-6, 22:4n-6, 24:6n-3, 24:5n-3; ∑n-3 PUFA is the sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 21:5n-3, 22:6n-3, 22:5n-3, 24:6n-3, 24:5n-3; ∑n-6 PUFA is the sum of 18:2n-6, 18:3n-6, 20:4n-6, 20:3n-6, 20:2n-6, 22:5n-6, 22:4n-6.

[c] FA not found (%total FA = 0) were 20:4n-3, 20:2n-6.

Table 4.6 Comparative mean percentage fatty compositions (%total fatty acids), standard error (SEM) and levels of significance (*P* values) between the heart, kidney and liver tissues in Australian dual-purpose lambs[a,b]

	Control		Medium		High		P value
Heart	Mean	SEM	Mean	SEM	Mean	SEM	
15:0	0.4	0.0^{A}	0.0	0.0^{B}	0.4	0.0^{A}	0.045
16:0	15.1	0.1^{A}	14.1	0.1^{B}	15.0	0.4^{A}	0.047
18:1n-9	18.1	0.4^{B}	19.8	0.1^{A}	19.0	0.1^{AB}	0.047
20:2n-6	0.0	0.0^{B}	0.1	0.0^{A}	0.0	0.0^{B}	0.006
Kidney	Mean	SEM	Mean	SEM	Mean	SEM	
22:6n-3	2.5^{B}	0.2	2.8	0.2^{A}	3.4	0.1^{A}	0.004
22:5n-6	0.1	0.1^{B}	0.9	0.0^{A}	0.5	0.1^{A}	0.011
∑SFA	46.6	1.6	44.4	0.9	42.7	1.3	0.035
Liver	Mean	SEM	Mean	SEM	Mean	SEM	
14:0	0.6	0.2^{A}	0.3	0.1^{B}	0.3	0.1^{B}	0.001
20:5n-3	1.0	0.6^{B}	3.5	0.5^{A}	2.8	0.6^{A}	0.048
20:3n-6	0.3	0.1^{B}	0.6	0.1^{A}	0.5	0.1^{A}	0.036
22:5n-6	0.1	0.0^{B}	1.1	0.0^{A}	0.1	0.0^{B}	0.010

[a] Means with different superscripts $^{A, B, C, D, E}$ within rows significantly differ ($P<0.05$).

[b] ∑SFA is the sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0.

Kidney fatty acid composition

In the kidney, the percentage of 22:5n-6 in the 10% and 20% supplementation groups were the highest (0.9% and 0.5% respectively) and significantly ($P<0.05$) differed from the control group (Table 4.4). Similarly, 22:6n-3 in the 20% supplementation group was significantly higher than those of the control and 10% supplementation groups (Tables 4.4 and 4.6).

Liver fatty acid composition

Within the liver of control and 10% supplementation treatment group of lambs, the highest 14:0 percentage (0.6%) was observed, which significantly ($P<0.01$) differed from the 0.3% in liver of lambs

in the 10% and 20% supplementation groups (Table 4.5). Percentages of 20:5n-3 in the 10% and 20% supplementation groups were significantly higher ($P<0.05$) than in the control group (Table 4.5). The highest percentage of 20:3n-6 was found in the 10% and 20% supplementation groups (0.6% and 0.5%, respectively). The highest 22:5n-6 content (1.1%) in liver was observed in the 10% *Spirulina* supplementation group, and this differed significantly from the other supplementation groups ($P<0.05$) (Tables 4.5 and 4.6).

Discussion

In this study, mRNA expression levels of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes were evaluated to provide greater insight into the effect of *Spirulina* supplementation on fatty acids and lipid metabolism in the heart, kidney, and liver organs of sheep. The most important finding from this study was the significant effects of different levels of *Spirulina* supplementation on the mRNA expression profiles of these four genes in the different organs. Our data indicate that genetic background, and to a lesser extent gender, determined the mRNA expression levels of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes. The *Spirulina* supplementation phase of the experiment demonstrated that the 10% level of *Spirulina* resulted in a higher PUFA composition in the tested organs.

The protein encoded by the *FASN* gene is a key lipogenic enzyme that catalyses all steps of the biosynthesis of long-chain FAs from an acetyl-CoA precursor (Berndt et al., 2007, Boizard et al., 1998). *FASN* is considered a fundamental enzyme in *de novo* synthesis step of lipogenesis, and its main function is to catalyse the synthesis of palmitate from acetyl-CoA and malonyl-CoA in the presence of NADPH into long-chain FAs (Byrne et al., 2005, Boizard et al., 1998). In the heart, *FASN* transcripts were significantly up-regulated in the 10% (low) supplementation group compared to the 0% (control) and 20% (high) supplementation groups. These findings are in line with the observed significant increases in 18:1n-9 (oleic acid) and 20:2n-6 (eicosadienoic acid) and a decrease of 16:0 (palmitic acid) in the

heart. Taken together, these findings suggest that the low level of *Spirulina* supplementation increases the transcription levels of the *FASN* gene in the heart, which might have contributed to an increase in n-6 and n-9 PUFA by utilizing palmitic acid, which is the first FA produced during FA synthesis as a precursor for longer FAs. This indicates that low *Spirulina* supplementation may result in a decrease in saturated FA and a concomitant increase in PUFA in the heart of supplemented lambs.

In the kidney, *AANAT* expression levels were significantly up-regulated in the 20% dietary *Spirulina* supplementation group compared to the 0% and 10% groups. Expression of the *AANAT* encoded protein accelerates the production of melatonin hormone by catalysing the rate-limiting step in the synthesis of melatonin from serotonin (Coon et al., 1999, Reiter et al., 2014). Melatonin is a hormone that controls the function of the circadian clock and therefore regulates activity and sleep (Coon et al., 1999, Reiter et al., 2014). Spanish scientists discovered that melatonin consumption stimulated browning of white fat tissue in rats (Jiménez-Aranda *et al.*, 2013). Brown fats burn, rather than store, calories. Thus, melatonin has an anti-obesity effect, and its metabolism protects against oxidative degradation of PUFA (Jiménez-Aranda et al., 2013, Reiter et al., 2014). These findings agree with our observations in the present study as both n-3 and n-6 PUFA levels increased and ∑SFA decreased in the kidney of lambs supplemented with 10% and 20% dietary *Spirulina*.

The *ADRB3* gene plays a key role in regulating mammalian energy storage and expenditure. It is also a principal mediator of the lipolytic and thermogenetic effects of high catecholamine (Forrest et al., 2007b, Wu et al., 2011a). The primary role of this receptor is in the regulation of resting metabolic rate and lipolysis (Forrest *et al.*, 2003). Herein, we demonstrated that *ADRB3* transcription levels were significantly up-regulated in the kidney of prime lambs supplemented with 20% dietary *Spirulina*, which is consistent with our observed FA results. This suggests that both the 10% and 20% levels of dietary *Spirulina* supplementation increased n-3 and n-6 PUFA compositions and decreased ∑SFA content in the kidney of lambs. However, in the liver, *ADRB3* transcripts were found to be differentially expressed

in the 10% compared to the 0% and 20% *Spirulina* groups. Given that the *ADRB3* gene encodes proteins regulating mammalian energy storage and expenditure by mediating effects from the sympathetic nervous system (Hu et al., 2010, Wu et al., 2012), our observations suggest an intricate genetics-nutrition interaction underpinning transcription at the molecular level that can be dietarily manipulated to achieve healthy FA composition outcomes. In addition, we demonstrated that *BTG2* transcription levels were significantly up-regulated in the liver of prime lambs supplemented with 20% dietary *Spirulina*.

B-cell translocation gene 2 belongs to the anti-proliferative gene family and has been shown to be involved in cell growth, differentiation, and survival (Mo *et al.*, 2011) as well as muscle fibre size, intramuscular fat deposition, and weight loss (Kamaid and Giráldez, 2008, Sasaki et al., 2006). FA analyses demonstrated that n-3 and n-6 FAs increased in the liver of lambs supplemented at 10% and 20% *Spirulina* levels. These findings are in line with the observed up-regulation of both *ADRB3* and *BTG2* transcriptions. This seems to suggest that dietary *Spirulina* supplementation increases metabolic rate and lipolysis in the liver through up-regulation of the *ADRB3* gene and simultaneously induces a decline in preadipocyte proliferation, an increase in energy expenditure, and a decline in energy uptake in adipocytes, ultimately enhancing n-3 and n-6 PUFA contents in the liver.

Conclusions

The results presented here demonstrated that mRNA expression levels of *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes in the heart, kidney, and liver are mainly influenced by dietary *Spirulina* supplementation level. Taken together, our results show that fatty acid metabolism in the kidney and liver are more sensitive to dietary manipulation than in the heart. These findings support the use of a low level of dietary *Spirulina* supplementation for an optimal increase in healthy n-3 and n-6 fatty acid contents of organs among Australian crossbred sheep.

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Chapter 5

Effects of *Spirulina* supplementation level on the expression of fat metabolism genes during simulated drought conditions in crossbred Australian sheep

Abstract

The effect of different *Spirulina* supplementation levels during simulated drought conditions on the mRNA expression of *Aralkylamine N-acetyltransferase* (*AANAT*), *Adrenergic beta-3 receptor* (*ADRB3*), *B-cell translocation gene 2* (*BTG2*), and *Fatty acid synthase* (*FASN*) genes in subcutaneous adipose tissue, *Longissimus dorsi* muscle, heart, kidney, and liver of Australian crossbred sheep were investigated. The hypothesis that supplementation with *Spirulina* will compensate for the drought condition feed loss and assist in increasing liveweight, growth, and body conformation was tested using purebred Merino weaners and first-cross weaners from merino dams sire-breed by Dorset and White Suffolk rams under the simulated drought condition. A total of twenty four lambs were randomly allocated into feeding trials that utilised lucerne hay basal diet. Each treatment group had six lambs balanced by sire-breed (Dorset, White Suffolk, Merino), sex (ewes, wethers), and *Spirulina* supplementation level (control: not supplemented, low: 50 ml, medium:100 ml, and high: 200ml). Lambs in low, medium, and high *Spirulina* treatment groups were drenched daily with *Spirulina.* Feeding trials lasted for nine weeks after three weeks of adjustment. Weekly measurements of *chest* girth (CG), wither height (WH), body length (BL), body condition score (BCS), and body live weight (BLW) were taken. After slaughter, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to assess the transcription levels of *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes in subcutaneous adipose, muscle, heart, kidney, and liver tissues. The evidence indicates that *Spirulina* supplementation resulted in a decrease of mRNA expression of *ADRB3* and *FASN* and an increase of *AANAT* genes mainly in subcutaneous and muscle tissues thought to be responsible for weight loss of lambs. In addition, a

decrease of mRNA expression of *BTG2* gene was shown to be responsible for higher energy expenditure and less fat deposition, exacerbating the lambs weight loss. These results are in the line and consistent with body condition and confirmation results suggesting that although supplementing with *Spirulina* has proved to increase lamb production and enhancement of polyunsaturated fatty acid (PUFA) of sheep meat, utilizing *Spirulina* as a supplement over drought condition may result in less lamb production and reduction in growth and weight loss.

Introduction

Diet has a major impact on the fatty acid composition and meat production of ruminants (Holman *et al.*, 2012). *Spirulina* is blue-green alga containing 60-70% protein, essential vitamins, and amino acids that has already been proven to increase lamb production (Holman *et al.*, 2012) and to reduce intramuscular fat percentage and lower the fat melting point under pasture-fed conditions (Holman, 2014). The protein-rich supplement, *Spirulina*, can be regarded as a suitable replacement for the traditional supplements such as cereals and oil. This suitability is due to several features that can make this microalga a promising supplement to lead current agriculture to a better condition in terms of quality and quantity (Holman *et al.*, 2014). *Spirulina* has a positive effect on the growth rate of lambs. The study by Holman *et al.* (2014) showed that this microalga improves the live-weights of the Australian dual-purpose lamb. Furthermore, several studies including the present study proved that *Spirulina* can impact several genes that contribute to the expression of fatty acids in order to make more products with better quality, where better quality is defined as having more n-3 and n-6 levels in this investigation.

Although in many parts of the world people are unaware of the importance of fatty acids in the content of meat and presume it as an unhealthy part of the meat, it can be said that fatty acids are important because it can define its quality in terms of nutritional value and flavour to the customers (Perez *et al.*, 2010). Polyunsaturated fatty acids (PUFA) such as n-3 polyunsaturated fatty acids (n-3 PUFA) and n-6

are known as the fatty acids that are recommended by scientists and nutritionists because of their nutritional value (Mapiye et al., 2011, Sasaki et al., 2006). It is suggested that they can reduce the possibility of some health issues such as cardiovascular disease, asthma, rheumatoid arthritis, cognitive decline, diabetes, osteoporosis, neurological dysfunction, and possible cancers (Nguyen et al., 2010, Alfaia et al., 2009). However, saturated fatty acids (SFA) and *trans* fatty acids are among the fatty acids that are known to be unhealthy (Mapiye et al., 2011, Sasaki et al., 2006). During the next chapters the different aspects of fatty acids (e.g., the important factor affecting its composition in the tissue or its amount in different animals) will be explained.

In previous studies, the mRNA expression of genes involved in fat metabolism was examined in order to fully understand the contribution of dietary supplementation of *Spirulina* to meat production and polyunsaturated fatty acid (PUFA) enhancement. In addition, the effect of dietary *Spirulina* under a drought condition and its potential to battle drought and enhance production needed to be investigated. The importance of *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes and their mRNA transcriptions for crossbred sheep production and PUFA enhancement have been already studied. Our experimental objective was to evaluate mRNA expression of the above genes under a simulated drought condition along with *Spirulina* supplementation and to examine the effect of dietary *Spirulina* on fat metabolism genes.

Materials and methods

Animal Welfare and Ethics Clearance

This study was approved by the University of Tasmania Animal Ethics Committee and conducted at the University of Tasmania Farm, Cambridge, Hobart, Australia. All procedures were conducted in accordance with 1993 Tasmanian Animal Welfare Act and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Experimental Design and Animal Management

Twenty-four weaned lambs from purebred Merino dams sired by White Suffolk, Dorset, and Merino rams lambs at 6 weeks of age were randomly assigned to four groups and were balanced by sire breed (White Suffolk, Dorset, Merino), sex (ewes, wether), and *Spirulina* supplementation level (control: not supplemented, low: 50 ml, medium: 100 ml, and high: 200ml). Lambs in low, medium, and high *Spirulina* treatment groups were drenched daily with *Spirulina.* Feeding trials lasted for nine weeks after three weeks of adjustment. *Spirulina* powder was purchased (TAAU, Darwin, Northern Territory, Australia) and converted into a water suspension using a 1:10 (w/v) ratio of *Spirulina* (g) to water (ml) and converted into percentage levels of 0, 5, 10 and 20%. Each lamb was directly provided with its assigned *Spirulina* supplementation level daily via oral drenching. All experimental lambs were slaughtered at the completion of each feeding trial at a commercial abattoir (Gretna Quality Meats, Gretna, Tasmania, Australia), except for the Merino-sired ewe lambs, which were saved for breeding purposes. Subcutaneous adipose, *longissimus dorsi* muscle, heart, kidney, and liver tissue samples were immediately removed from each carcass, frozen in liquid nitrogen, and transported to the laboratory, where they were stored at -80 °C until RNA extraction and further analyses.

Liveweight and body confirmation measurements

At weekly intervals, each lamb was individually assessed for chest girth (CG), wither height (WH), body length (BL), body condition score (BCS), and body live-weight (BWT) measurements.

RNA extraction and cDNA synthesis

Total RNA was isolated from frozen tissue using the TRIzol® Plus RNA Purification Kit (Life Technologies Pty Ltd. Victoria, Australia). Homogenisation of the sample in TRIzol® Reagent was performed using a tissue lyser (Qiagen Ltd., Crawley, UK), and the RNA was subsequently extracted using chloroform and precipitated using isopropanol. Quantity and quality of total RNA was assessed using the NanoDrop 8000 spectrophotometer (NanoDrop, Wilmington, DE). RNA quality was verified by ensuring all RNA samples had an absorbance (A260/280) of between 1.8 and 2. RNA samples were

treated with PureLink^{TM}DNase (Life Technologies Pty Ltd. Victoria, Australia) and purified using the RNeasy1 mini kit (Qiagen Ltd.). DNase-treated and purified total RNA was then reverse transcribed to cDNA, with Mixed Oligo dT/Random Hexamer Primers, using the Tetro cDNA Synthesis Kit (Bioline Pty Ltd. NSW, Australia) according to the manufacturer's instructions and stored at -80 °C for subsequent analyses.

Primer design and reference gene selection

All candidate and reference gene primers used to detect gene expression (Table 6.1) in this study were designed using the Primer3 web based software program (http://frodo.wi.mit.edu/primer3/) and obtained from a commercial supplier (GeneWorks Pty Ltd., SA, Australia).

Table 5.1 Primer pairs designed for quantitative real-time PCR (qRT-PCR)

[a]Gene symbol	qPCR Primers		[b]T_a	Amplicon Size (bp)
	Forward Primer	Reverse Primer		
AANAT	ACTGACCTTCACGGAGATGC	TTCACTCATTCTCCCCGTTC	60	211
ADRB3	TCAGTAGGAAGCGGGTCGGG	GGCTGGGGAAGGGCAGAGTT	60	291
BTG2	CTGGAGGAGAACTGGCTGTC	AAAACAATGCCCAAGGTCTG	60	194
FASN	GTGTGGTACAGCCCCTCAAG	ACGCACCTGAATGACCACTT	60	110
Reference genes				
UBC	CGTCTTAGGGGTGGCTGTTA	AAATTGGGGTAAATGGCTAGA	60	90
PPIA	TCATTTGCACTGCCAAGACTG	TCATGCCCTCTTTCACTTTGC	60	72

[a]Aralkylamine N-acetyltransferase=AANAT, *β*3-adrenergic receptor=ADRB3, B-cell translocation gene 2=BTG2, Fatty acid synthase=FASN, Ubiquitin C=UBC, Peptidyl-prolyl cis-trans isomerasa=PPIA, [b]Ta=Empirical annealing Temperature.

Primer specificity was checked using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/). All primers were validated using a pooled cDNA sample. A standard curve was generated using serial dilutions of pooled cDNA. PCR products generated by amplification were sequenced to verify their primer specific identity (Beckman Coulter CEQ^{TM} 8000 Series Genetic Analysis System, University Tasmania). To determine the relative gene expression levels, suitable highly stable reference genes were required. In the current study, out of five tested, two reference genes—*Ubiquitin C* (*UBC*) and *Peptidyl-prolyl cis-trans isomerasa* (*PPIA*)—were used to normalise gene expression data for all *Aralkylamine N-acetyltransferase* (*AANAT*), *NGF-inducible anti-proliferative protein PC3* (*BTG2*), *Fatty acid synthase* (*FASN*), and *Beta-3 Adrenergic Receptor* (*ADRB3*) genes and tissues. The principle behind the selection of the reference gene is that the expression ratio of two perfect reference genes should be constant across all samples. The expression stability of the reference genes was validated with the software program, geNorm version 3.5, by calculating the gene expression stability measure (M value).

Quantitative real time PCR (qPCR)

Following reverse transcription, cDNA quantity was determined and standardised to the required concentration for qPCR. Triplicate 20 μL reactions were carried out in 72-well Rotor-Gene (QIAGEN GmbH, Hilden, Germany) containing 4 μL cDNA (50 ng), 10 μL 2x SensiFAST SYBR No-ROX Mix (Bioline Pty Ltd., NSW, Australia), 4.4 μL DEPC H2O, and 0.8 μL forward and reverse primers (100 fmol). Assays were performed using the Rotor-Gene 3000 (QIAGEN Pty Ltd., VIC, Australia) with the following cycling parameter rs: 95 C for 2 min polymerase activation and 40 cycles of 95 C for 5 s denaturation, 60 C for 10 s annealing followed by 72 C for 5 s extension. Gene expression levels were recorded as Ct values, i.e., the number of PCR cycles at which the fluorescence signal is detected above the threshold value. All samples were run in triplicate. Amplification efficiencies were determined for all candidate and reference genes using the formula E=10^(−1/slope), with the slope of the linear curve of cycle threshold (Ct) values plotted against the log dilution (Higuchi *et al.*, 1993). Primer

concentrations were optimised for each gene, and disassociation curves were examined for the presence of a single PCR product. The efficiency of the reaction was calculated using a 5-fold serial dilution of cDNA and generation of a standard curve. All PCR efficiency coefficients were between 1.7 and 1.8, and therefore they were deemed acceptable. The software package Rotor-Gene 3000 version 6.0.16 software (QIAGEN Pty Ltd., VIC, Australia) was used for efficiency correction of the raw Ct values, inter-plate calibration based on a calibrator sample included on all plates, averaging of replicates, normalisation to the reference gene and the calculation of quantities relative to the highest Ct, and log2 transformation of the expression values for all genes.

Statistical analyses

Factorial ANOVA (PROC GLM) and Mixed Model (PROC MIXED) (SAS Institute, 2009) procedures were used to fit the fixed effects of *Spirulina* level on BWT, ADG, CG, WH, BL, and BCS. Separation of mean differences using Duncan's multiple range tests and Bonferroni pairwise comparison tests was conducted at a $P<0.05$ level of significance. Pearson's correlation coefficients (PROC CORR) between dependent variables were also estimated, and significance was established using Bonferroni tests (SAS, 2009). A generalised linear model (GLM) (SAS Inst., NC) was used in computing the fixed effects of *Spirulina* supplementation level, sire-breed and sex, and their interactions on mRNA expression level of *AANAT*, *ADRB3*, *BTG2,* and *FASN* genes in subcutaneous adipose, muscle, heart, kidney, and liver.

Results

Spirulina supplementation during stimulated drought condition and phenotypic data

Spirulina supplementation caused sheep to have smaller CG than the control group ($P<0.0001$) (Table 5.2).

Table 5.2 Least square means (LSM) of chest girth, wether height, body length, body condition score, live weight and average daily gain in *Spirulina* supplemented crossbred lambs during a stimulated drought condition

	Spirulina				**P Values**
	0	5%	10%	20%	*Spirulina*
GC (cm)	88.67 ± 0.47	85.82 ± 0.59	86.32 ± 0.52	86.98 ± 0.42	<0.0001***
WH (cm)	64.43 ± 0.51	63.85 ± 0.45	64.17 ± 0.50	64.32 ± 0.42	0.7234^{ns}
BL (cm)	70.90 ± 0.68	69.45 ± 0.58	68.90 ± 0.47	68.07 ± 0.43	<0.0001***
BCS (0-5)	3.72 ± 0.08	3.43 ± 0.08	3.43 ± 0.08	3.43 ± 0.07	<0.0001***
BWT (kg)	38.18 ± 0.88	36.46 ± 0.71	35.62 ± 0.84	37.47 ± 0.66	<0.0001***
ADG (kg/d)	-0.01 ± 0.04	0.01 ± 0.04	0.00 ± 0.04	-0.00 ± 0.04	0.9850^{ns}

Column means within a fixed effect bearing different superscripts significantly differ (P<0.05). Chest girth (CG), withers height (WH), body length (BL), body condition score (BCS), body weight (BWT), and average daily weight gain (ADG). Level of significance: ns not significant (P>0.05), * significant (P<0.05), ** highly significant (P<0.01), and *** very highly significant (P<0.001).

Furthermore, sheep receiving *Spirulina* supplementation had shorter bodies (BL) than the control group (P<0.0001). Sheep fed with *Spirulina* had lower BCS than the control group (P<0.0001) (Table 5.2). It was observed that sheep receiving *Spirulina* supplementation under simulated drought condition statistically significantly lost weight (P<0.0001). The 10% *Spirulina* supplementation group showed the lowest BWT of 35.62 between all treatments (Table 5.2). No differences in WH and ADG between control and treatment groups were observed. The phenotypic results are shown in Table 5.2.

Gene expression pattern

To determine the expression pattern of *AANAT, ADRB3, BTG2,* and *FASN* genes, a panel of tissue were collected from 20 genetically divergent Australian crossbred sheep namely White Suffolk, Dorset, and Merino. The genes were investigated directly for mRNA expression by qRT-PCR using the subcutaneous adipose, muscle, heart, kidney, and liver tissue of sheep fed a diet supplemented with 0, 5, 10, or 20% *Spirulina* during a simulated drought condition. The qRT-PCR results were calibrated and

normalised using two housekeeping genes (UBC and PPIA) and the qBase relative quantification excel application (Pfaffl, 2001) for automated analysis.

Gene expression in subcutaneous adipose tissue

The relative mRNA expression levels of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes analysed in the subcutaneous adipose tissue are presented in Figure 5.1.

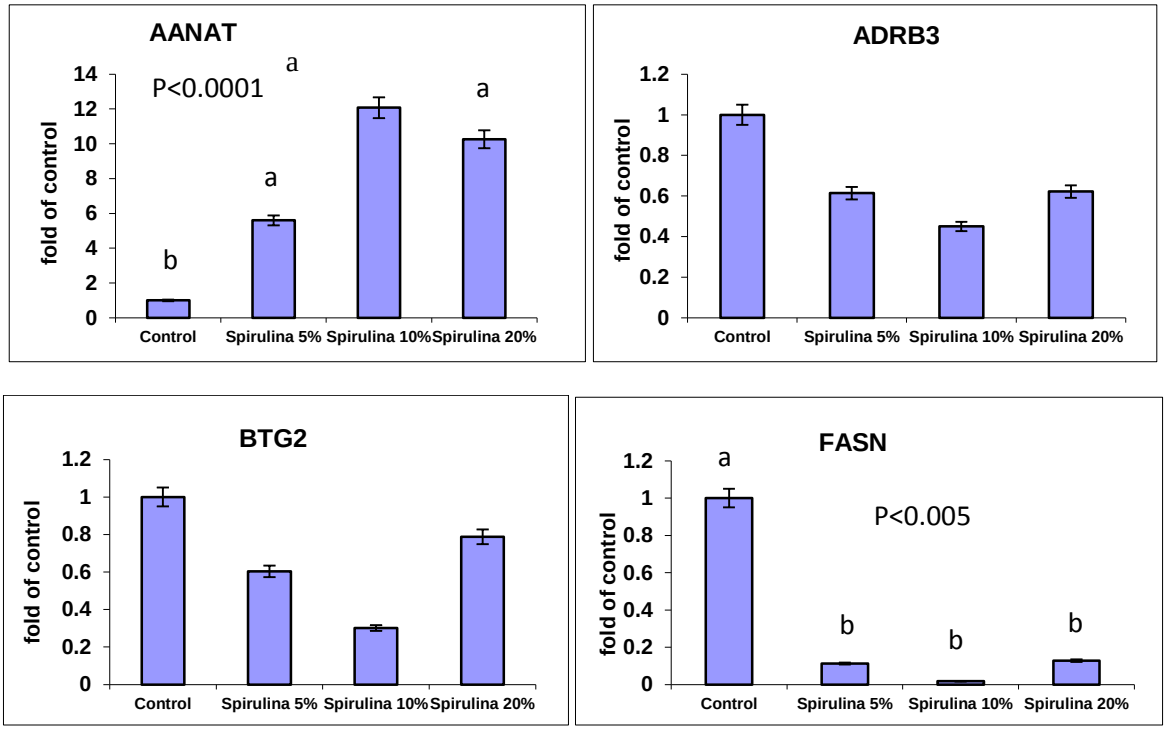

Figure 5.1 Relative expression levels of AANAT, ADRB3, BTG2, and FASN genes in the subcutaneous adipose tissue (SAT) of Australian crossbred sheep fed 0, 5, 10, and 20% *Spirulina* supplementation during a simulated drought condition. Each value was normalised to *UBC* and *PPIA* expressions. Least square means with different superscripts differ by at least $P<0.05$

Aralkylamine N-acetyltransferase showed higher expression levels in tissues from sheep that received 5, 10, and 20% *Spirulina* supplement relative to 0% *Spirulina*. The 5, 10, and 20% dietary *Spirulina* corresponded to 5.59, 12.07, and 10.25 *AANAT* mRNA fold changes, respectively. In contrast, *Spirulina* supplementation did not alter the mRNA expression of *ADRB3* and *BTG2* genes in subcutaneous adipose. *FASN* mRNA expression showed less expression levels in tissues from sheep that received 5,

10 ,and 20% *Spirulina* supplement relative to 0% *Spirulina*. The 5, 10, and 20% of dietary *Spirulina* supplementation corresponded to 0.11, 0.01, and 0.12 fold changes compared to the control group.

Gene expression in longissimus dorsi (ls) muscle tissue

The relative mRNA expression levels of the four genes analysed in *longissimus dorsi* (*ls*) muscle tissues are presented in Figure 5.2.

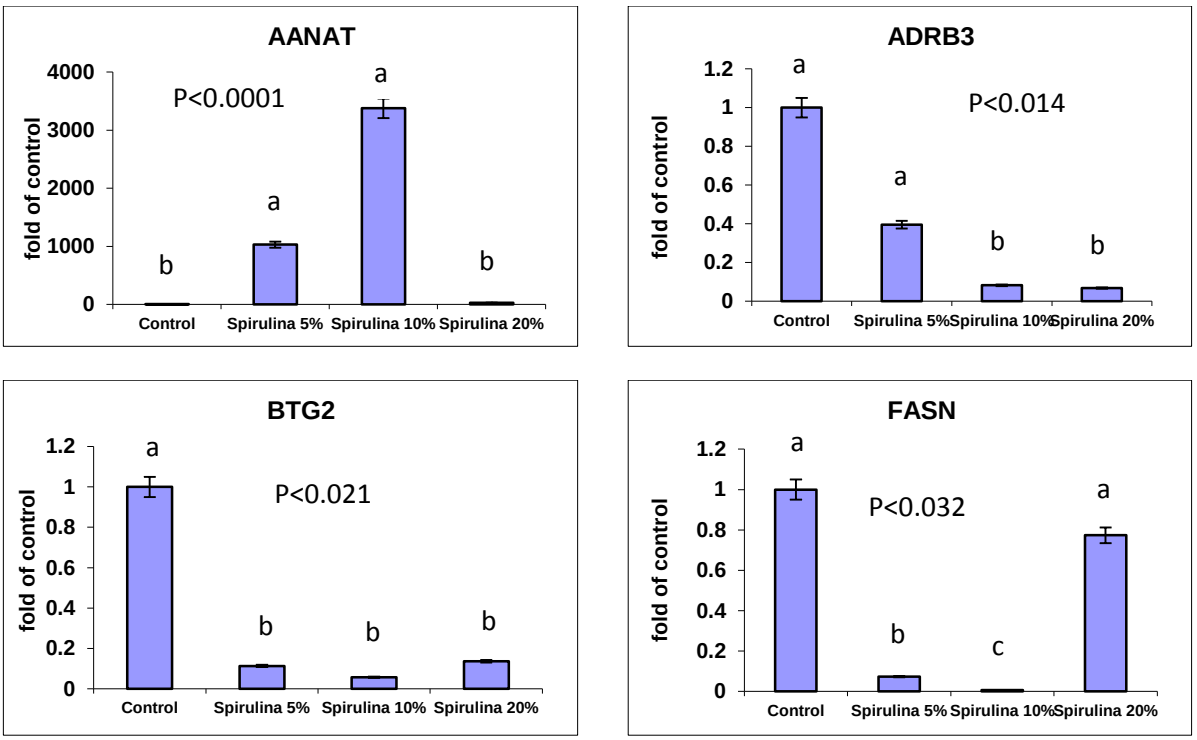

Figure 5.2 Relative expression levels of AANAT, ADRB3, BTG2, and FASN genes in the *longissimus dorsi* (*ls*) muscle of Australian crossbred sheep fed 0, 5, 10, and 20% *Spirulina* supplementation during a simulated drought condition. Each value was normalised to *UBC* and *PPIA* expressions. Least square means with different superscripts differ by at least $P<0.05$

It is evident that *AANAT* mRNA showed higher expression levels in tissues from sheep that received 5, 10, and 20% *Spirulina* relative to the control group. The 5 and 10% dietary supplementation corresponded to 1030 and 3377 of *AANAT* mRNA fold changes compared to the control group's muscle tissue, respectively. In contrast, 10 and 20% *Spirulina* supplementation levels showed less mRNA expression of the ADRB3 gene as 0.08 and 0.06 fold changes compared to the control group, respectively. *Spirulina* supplementation showed less BTG2 mRNA expression levels relative to 0%

Spirulina. The 5, 10, and 20% of dietary *Spirulina* corresponded to 0.11, 0.05, and 0.13 of fold changes, respectively. *FASN* showed lower expression levels in tissues from sheep that received 5 and 10% *Spirulina* supplementation. The 5 and 10% dietary *Spirulina* corresponded to 0.07 and 0.006 fold changes, respectively.

Gene expression in heart tissue

The relative mRNA expression levels of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes analysed in the heart tissue are presented in Figure 5.3.

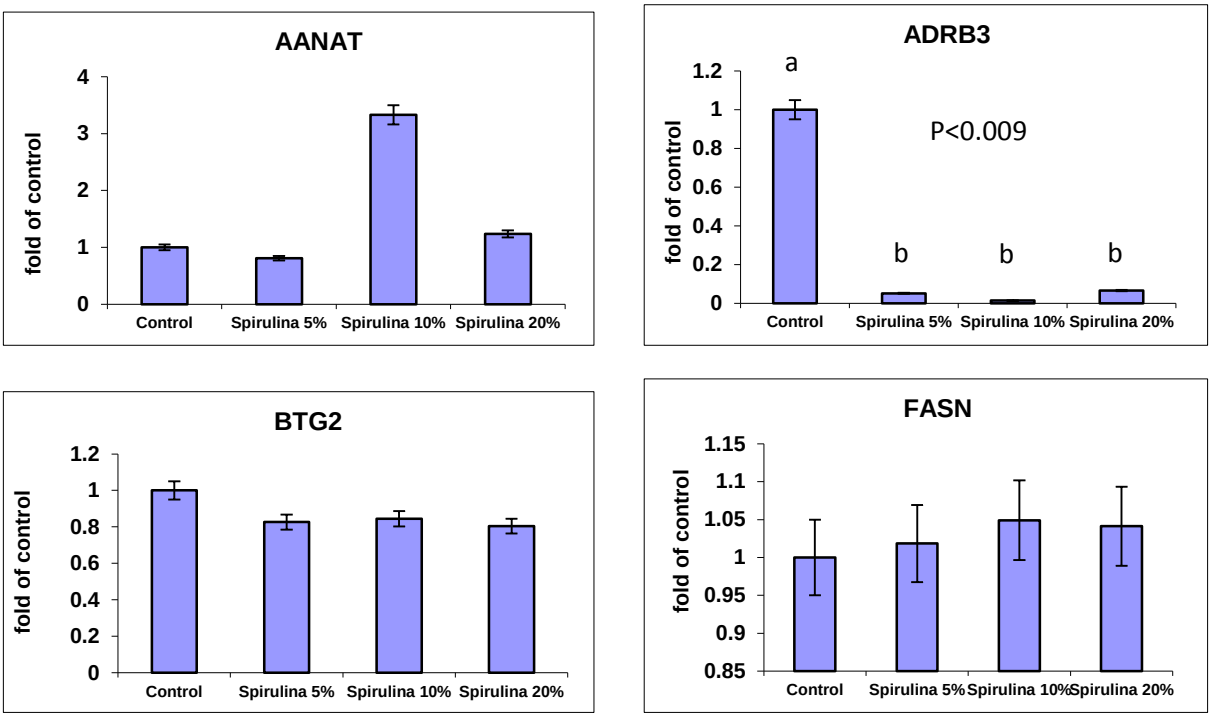

Figure 5.3 Relative expression levels of AANAT, ADRB3, BTG2, and FASN genes in the heart of Australian crossbred sheep fed 0, 5, 10, or 20% *Spirulina* supplementation during a simulated drought condition. Each value was normalised to *UBC* and *PPIA* expressions. Least square means with different superscripts differ by at least *P*<0.05

Adrenergic beta-3 receptor expression was lower in the tissues of sheep that received *Spirulina* supplementations compared to sheep that received 5, 10, and 20% *Spirulina*. The dietary *Spirulina* supplementation significantly down-regulated the mRNA expression of *ADRB*3 gene (P<0.009), corresponding to 0.05, 0.01, and 0.06 fold changes compared to the control group. In contrast, *Spirulina* supplementation levels did not change the mRNA expression of the *AANAT*, *BTG2*, and *FASN* gene in heart tissues.

Gene expression in kidney tissue

The relative mRNA expression levels of the four genes analysed in kidney tissues are presented in Figure 5.4.

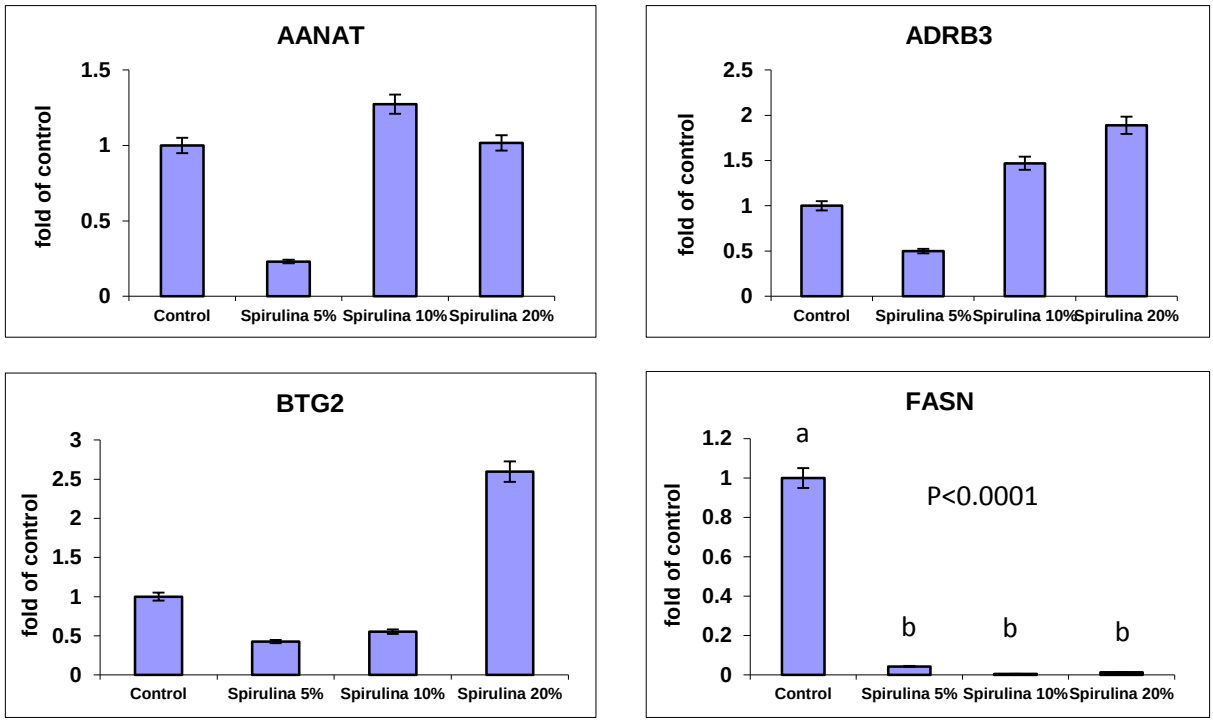

Figure 5.4 Relative expression levels of AANAT, ADRB3, BTG2, and FASN genes in the kidney of Australian crossbred sheep fed 0, 5, 10, or 20% *Spirulina* supplementation during a simulated drought condition. Each value was normalised to *UBC* and *PPIA* expressions. Least square means with different superscripts differ by at least $P<0.05$

In kidney tissues, *Spirulina* supplementation significantly (P<0.0001) down-regulated the mRNA expression of the *FASN* gene under all supplementation levels. The 5, 10, and 20% dietary *Spirulina* corresponded to 0.04, 0.004, and 0.01 fold changes, respectively. *Spirulina* supplementation did not alter the mRNA expression of the *AANAT*, *ADRB3,* and *BTG2* genes in kidney tissues.

Gene expression in liver tissue

The relative mRNA expression in levels of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes analysed in the liver tissues are presented in Figure 4.5.

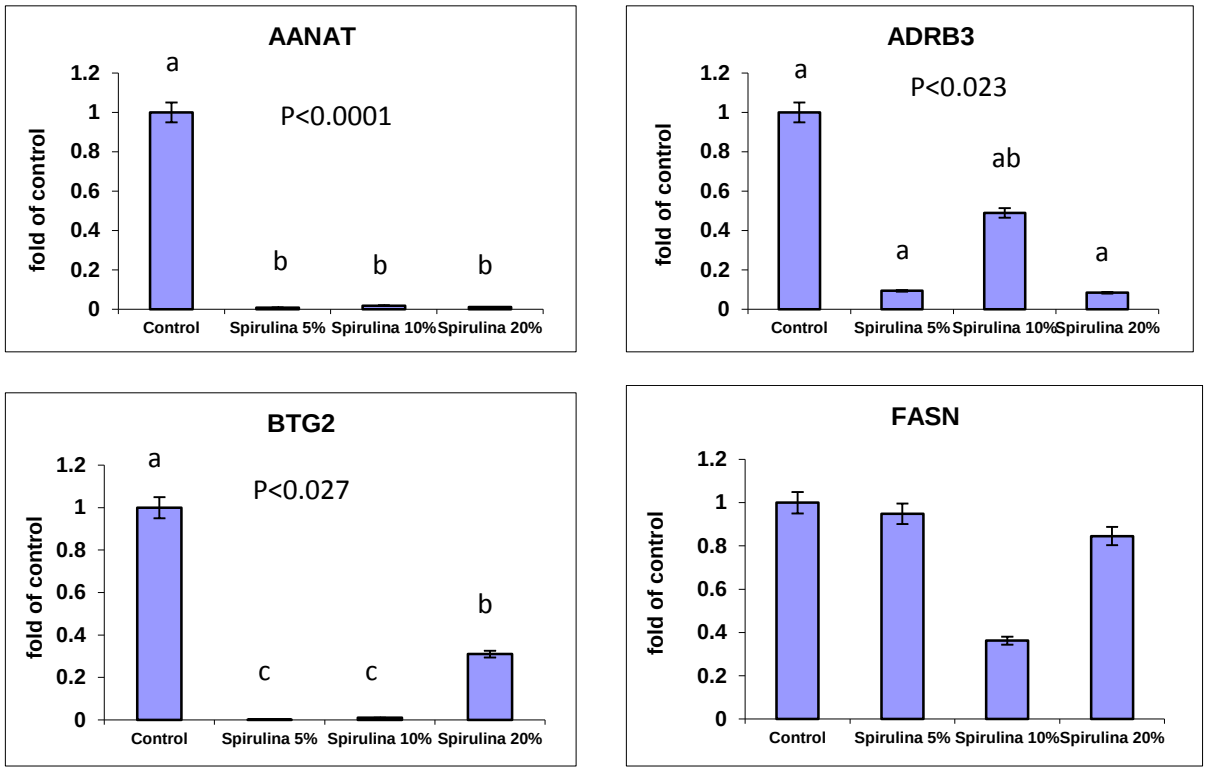

Figure 5.5 Relative expression levels of AANAT, ADRB3, BTG2, and FASN genes in the liver of Australian crossbred sheep fed 0, 5, 10, or 20% *Spirulina* supplementation during a simulated drought condition. Each value was normalised to *UBC* and *PPIA* expressions. Least square means with different superscripts differ by at least *P*<0.05

Lower *AANAT, ADRB3,* and *BTG2* mRNA expression levels were detected in liver tissues from sheep receiving *Spirulina* supplementations relative to the control treatments. The 5, 10, and 20% dietary *Spirulina* corresponded to 0.007, 0.01, and 0.01 fold changes of *AANAT* mRNA, respectively. The same dietary *Spirulina* corresponded to 0.09, 0.48, and 0.08 fold changes of *ADRB3* mRNA, respectively. The 5, 10, and 20% *Spirulina* supplementation levels corresponded to 0.0009, 0.009, and 0.30 fold changes of *BTG2* mRNA in liver tissues, respectively. In contrast, dietary *Spirulina* did not alter the mRNA expression of the *FASN* gene in the liver tissues.

Discussion

Previous studies have indicated that the use of 10% *Spirulina* as a dietary supplement under pasture-fed management can lower the intramuscular fat percentage (IMF) and fat melting points (FMP), suggesting

a possible increase in unsaturated fatty acids (UFA) and a decrease in saturated fatty acids (SFA) in crossbred Australian sheep meat (Holman *et al.*, 2012). Furthermore, 10% *Spirulina* supplementation under a pasture-fed condition has proven to enable sheep to grow longer bodies and increase their body live-weight (Holman *et al.*, 2012). Further gene expression investigation of the genes involved in fat metabolism has presented that mainly in both subcutaneous adipose and muscle tissues, 10% *Spirulina* supplementation under a pasture-fed condition has increased the mRNA expression of *ADRB3* and *FASN* genes, which are mainly involved in fat metabolism. The basic function of the *ADRB3* gene is energy balance by thermoregulation and lipolysis (Hu et al., 2010, Wu et al., 2012). The encoded enzyme by the *FASN* gene is a multifunctional enzyme that catalyses the synthesis of fatty acids (Berndt *et al.*, 2007). In addition, it was shown that the mRNA expression of the *AANAT* gene, which is responsible for melatonin production, was increased under *Spirulina* supplementation. Melatonin is the hormone that regulates the night/day rhythm and is synthesized by a supplementary protein that is encoded by *AANAT*. Melatonin can also lead to weight loss by stimulating the production of brown fat through the browning process of white fat tissue and PUFA enhancement (Coon et al., 1999, Perez et al., 2010, Reiter et al., 2014). These together with the observed increase of mRNA expression of the *BTG2* gene in the tissues receiving dietary *Spirulina* suggested the possible application of 10% *Spirulina* supplementation under a pasture-fed condition to both increase lamb production and enhance the polyunsaturated fatty acid (PUFA) profile, especially n-3 and n-6.

However, the use of dietary *Spirulina* under drought conditions and its effects on the mRNA expression of genes involved in fat metabolism and n-3 enhancement has not been fully studied. In this study, *Spirulina* supplementation under a simulated drought condition caused sheep to lose weight and decreased the body measurements factors. In other words, *Spirulina* dietary supplementation, and especially the 10% level of supplementation, decreased crossbred lamb production under drought conditions. One possible explanation for this observation could be a lack of good quality pasture and fibre in the animals' diet. In addition, the excessively high dietary protein intake is proven to suppress

optimal sheep growth (Holman *et al.*, 2012). This is thought to be due to the negative correlation between protein accretion and fat deposition rates, the latter being exacerbated by high feed protein levels (Holman *et al.*, 2012). This was shown in a previous study, as 20% dietary *Spirulina* decreased the live-weight of Australian crossbred sheep under a pasture-fed condition (Holman *et al.*, 2012). In this study, the live-weight and body measurements indicated that lamb productivity decreased under a simulated drought condition due to the low feed quality. Furthermore, *Spirulina* supplementation exacerbated this loss and proved to not be a good alternative for feed loss. The observed mRNA transcription of the *AANAT*, *ADRB3*, *BTG2*, and *FASN* gene expression levels are also consistent with the observed phenotypic data indicating that the reductions in fat deposition, especially in subcutaneous adipose and muscle tissues, were related to the significant decrease of *FASN* and *ADRB3* mRNA expression levels in tissues receiving *Spirulina* supplementation. Adrenergic beta-3 receptor and *FASN* genes are responsible for fat synthesis; therefore, not only did dietary *Spirulina* not compensate the feed loss, but it also reduced their transcription levels leading to production losses. On the other hand, the increase of the *AANAT* transcriptions indicated a possible increase of melatonin production, which is responsible for weight loss, in both subcutaneous adipose and muscle tissues. The observed decreases in transcription levels of *BTG2* suggest extra energy expenditure and less fat deposition in lamb tissues receiving dietary *Spirulina* supplement. These findings indicate that the dietary *Spirulina cannot* solely compensate feed loss, and it should be utilized with good quality feed to both increase lamb production and PUFA enhancement.

Chapter 6

Effect of level of *Spirulina* supplementation on the fatty acid compositions of adipose, muscle, heart, kidney, and liver tissues in Australian dual-purpose lambs

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Abstract

This study investigated the effects of *Spirulina* supplementation on the fatty acid (FA) compositions of subcutaneous adipose, longissimus dorsi muscle, kidney, heart, and liver tissues in crossbred Australian sheep. *Spirulina* powder was dissolved in water using a *Spirulina* to water ratio of 1 g: 10 mL. Forty lambs sired by Black Suffolk, White Suffolk, Dorset and Merino rams were assigned into 4 treatment groups of *Spirulina* supplementation levels comprising the following groups: control (0 mL), low (50 mL), medium (100 mL), and high (200 mL) referred to as the 0, 5, 10, and 20% groups. Each lamb was orally drenched with either water (control group) or *Spirulina* daily before being released for grazing. Supplementation continued over a 9-week feeding trial duration, consisting of a 3-week adjustment phase and 6-week experimental period. All experimental lambs were run together as a single mob with *ad libitum* access to drinking water and a basal diet of ryegrass pasture and cracked barley. The lambs

were slaughtered after 9 weeks of supplementation and heart, kidney, adipose, liver, and muscle tissue samples were collected. FA profiles obtained by gas chromatography were transformed to percentages of total FA and analysed in SAS using general linear model procedures of fitting tissue, level of supplementation, sire breed, and sex as fixed effects. The results demonstrated significant variations in growth and body conformation traits and tissue and organ FA composition in response to the *Spirulina* diet. The medium-level (10%) *Spirulina* diet increased the n-3 and n-6 polyunsaturated fatty acid (PUFA) composition in all tissues and organs significantly ($P<0.05$ and $P< 0.01$). We suggest the use of medium level (10%) of *Spirulina* supplementation in order to increase lamb production with more n-3 and n-6 PUFA and therefore higher nutritional quality.

Keywords: *Spirulina,* Fatty acids, Sheep, n-3, n-6, heart, liver, kidney, muscle, adipose tissues

Introduction

Spirulina platensis is a cyanobacterium (blue-green alga) commercially produced as a nutritional supplement for humans and as a feed ingredient for livestock (Ciferri, 1983, Qureshi et al., 1996, Upasani et al., 2001). Spirulina is produced mainly from two species of cyanobacteria: Arthrospira platensis and Arthrospira maxima. Spirulina platensis has a high content of protein (60%), essential vitamins, and minerals. Thus, it is regarded as a desirable nutrient supplement (Belay et al., 1993, Ciferri, 1983, Ross and Dominy, 1990).

In the dual-purpose prime lamb industry, accomplishing a higher quality of lamb product is central to achieving economic success. When it comes to making purchasing decisions, carcass characteristics and meat quality are significant standards for the industry and consumers (Alfaia et al., 2009, Kouba and Mourot, 2011, Smet et al., 2004). In meat, the fatty acid composition and cholesterol levels have received considerable attention due to their significance in human health and product quality (Mapiye et al., 2011,

Woods and Fearon, 2009). There are two types of factors that influence both the quantity and quality of lipids in animal products: internal factors such as age, genotype, and gender, and external factors such as feeding and temperature.

In recent years, there has been increased research interest in manipulating the fatty acid composition of meat. Nowadays, consumers ask for natural and healthy products. Thus, there is great interest in research to produce foods of higher nutritional quality, including meats (Mapiye et al., 2011, Moibi and Christopherson, 2001, Woods and Fearon, 2009). Modification of animal diets using bioactive feed supplements such as *Spirulina* is one strategy for producing such foods (Doreau et al., 2010, Iwata et al., 1990).

The major objective of this study is to investigate the effect of the level of *Spirulina* supplementation on the fatty acid (FA) composition of subcutaneous adipose, *longissimus dorsi* muscle, heart, kidney, and liver tissues in dual-purpose Australian lambs. It was hypothesized that *Spirulina* would affect lamb tissues and organ FA composition.

Materials and Methods

This study was carried out at the University of Tasmania (UTAS) Farm, Cambridge, Tasmania, Australia. All experimental processes conformed to the UTAS Animal Ethics Committee guidelines, the 1993 Tasmania Animal Welfare Act, and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Animal management, slaughter, and sampling

At the UTAS Farm, Merino, Dorset, Black Suffolk, and White Suffolk terminal sires were mated over two consecutive years with purebred Merino ewes using a 1:100 ram-to-ewe mating ratio. All first

progeny were identified using the National Livestock Identification ear tags. All lambs were weaned onto ryegrass pastures at three months of age. At 6 months of age, 48 lambs were allocated into a feeding trial using either ryegrass pasture or simulated drought Lucerne hay. Each treatment group had eight lambs balanced by sire breed (Black Suffolk, White Suffolk, Dorset, and Merinos), sex (ewe, wether), and *Spirulina* supplementation level (control: no supplementation, low: 50 ml/5%, medium: 100 ml/10%, and high: 200 ml/20%).

Spirulina powder was purchased (TAAU, Darwin, Northern Territory, Australia) and made into a water suspension using a 1:10 w/v ratio of *Spirulina* (g) to water (ml) and converted into percentage levels of 0%, 5%, 10%, and 20%. Each lamb was directly provided with its assigned *Spirulina* supplementation level daily via oral drenching. All experimental lambs were slaughtered at the completion of each feeding trial at a commercial abattoir (Gretna Quality Meats, Gretna, Tasmania, Australia), except for the Merino-sired ewe lambs, which were saved for breeding purposes. Subcutaneous adipose deposit, kidney, liver, heart, and *longissimus dorsi* muscle tissue samples were immediately removed from each carcass and frozen in liquid nitrogen, transported to the laboratory, and stored at -20 °C for further analysis.

Lipid extraction and GC analysis

All tissue samples (subcutaneous adipose, kidney, heart, liver, and muscle) were extracted using a modified Bligh and Dyer protocol (Bligh and Dyer, 1959). This involved a single-phase overnight extraction using $CHCl_3$: MeOH: H2O (1:2:0.8 v/v), followed by phase separation with the addition of $CHCl_3$:salineH_2O (1:1 v/v). The total lipid extract was obtained by rotary evaporation of the lower chloroform phase.

An aliquot of total lipid extracted from each sample was transmethylated in MeOH:$CHCl_3$:HCl (10:1:1 v/v) for 2 h at 80 °C. Milli-Q H_2O (1 ml) was then added before FA methyl esters (FAME) were extracted

with hexane: chloroform (4:1 v/v) and reduced under a nitrogen stream, and a known concentration of an internal injection standard (19:0 FAME) was added. An Agilent Technologies 7890B gas chromatograph (GC) (Palo Alto, California USA) equipped with an Equity™-1 fused silica capillary column (15 m x 0.1 mm internal diameter and 0.1-µm film thickness), a flame ionisation detector, a split/splitless injector, and an Agilent Technologies 7683 B Series autosampler was used in the analysis.

Samples were injected in splitless mode and were carried by helium gas at an oven temperature of 120 °C. Post injection, the oven temperature was increased to 270 °C at 10 °C/min, and then to 310 °C at 5 °C/min. Peaks were quantified by Agilent Technologies ChemStation software (Palo Alto, California USA). FA identities were confirmed using GC-mass spectrometric (GC/MS) analysis. These analyses were performed using a Finnigan Thermoquest GCQ GC-MS fitted with an on-column injector with Thermoquest Xcalibur software (Austin, Texas USA). The GC had an HP-5 cross-linked methyl silicone-fused silica capillary column (50 m x 0.32 mm internal diameter). The carrier gas used was helium, with operating conditions previously described (Miller *et al.*, 2006).

Statistical analysis

Individual FAs and the summations of saturated, monounsaturated, and polyunsaturated FAs were computed as percentages of total FAs. 'Statistical Analysis System' software (SAS Institute., 2009) was used to analyse the data, and summary statistics were computed with means, standard deviations, and minimum and maximum values to check for errors and outliers. Repeated measures analysis of variance in general linear models procedure (PROC GLM) using Statistical Analysis System (SAS Institute., 2009) was then carried out by fitting the level of *Spirulina* supplementation, sire breed, sex, and tissue as fixed effects in the model and using FA values as dependent variables (14:0, 15:0, 16:1n-9c, 16:1n-7c, 16:0, 17:0, 18:2n-6, 18:3n-3, 18:1n-9, 18:1n-7c, 18:1n-7t, 18:0, 20:4n-6, 20:5n-3, 20:3n-6, 20:4n-3, 20:2n-6, 20:0, 22:5n-6, 22:6n-3, 22:5n-3, 22:0, 23:0, 24:0, ∑SFA, ∑MUFA, ∑PUFA, Σ n-3 PUFA, Σ n-6 PUFA).

Bonferroni's probability pairwise comparison test was used to separate mean differences, and the level of significance was defined as $P<0.05$.

Results

Spirulina supplementation and phenotypic data

Spirulina supplementation enabled sheep to grow longer bodies (BL) than the control group (P<0.015). Furthermore, lambs in the high (20%) Spirulina supplementation treatment group had a greater body condition score (BCS) than the medium (10%), low (5%), and control (0%) treatment groups (P<0.001). It was observed that the sheep receiving medium Spirulina supplementation had the greatest body weight (BWT) of 41.9 kg (P<0.018), but no differences between the high, low, and control treatment groups were observed. The phenotypic results are shown in Table 6.2.

Table 6.1 The fatty acid composition of *Spirulina* analysed by GC-MS

Spirulina (% total FA)	Mean	SEM
16:0 Palmitic acid	24.8	1.4
16:1n-9c Palmitoleic acid	3.7	0.5
17:0 Heptadecanoic acid, or margaric acid	1.7	0.1
18:0	6.3	0.9
18:1n-9 Oleic acid	9.8	1.1
18:2n-6 Linoleic acid	12.2	1.4
18:3n-3 α-Linolenic acid	4.46	0.3
20:0 Arachidic acid	2.1	0.2
20:2n-6 Eicosadienoic acid	1.9	0.4
20:3n-6 Dihomo-γ-linolenic acid (DGLA)	2.2	0.2
20:5n-3 Eicosapentaenoic acid	1.95	0.1

Table 6.2 Least square means (LSM) of chest girth, wether height, body length, body condition score, live weight, and average daily gain in *Spirulina*-supplemented crossbred lambs.

Spirulina levels									
	0		5%		10%		20%		P value
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
GC (cm)	95.0	0.6	95.2	0.4	95.6	0.6	96.1	0.7	0.376^{ns}
WH (cm)	62.9	0.4	62.5	0.6	62.7	0.4	63.1	0.3	0.669^{ns}
BL (cm)	65.7^{b}	0.4^{b}	65.6	0.4^{b}	66.6	0.4^{a}	66.8	0.4^{a}	0.015*
BCS (0-5)	3.2^{b}	0.1^{b}	3.1	0.1^{b}	3.3	0.0^{b}	3.4	0.1^{a}	0.001^{***}
BWT (kg)	40.6^{b}	0.7^{b}	40.7	0.4^{b}	41.9	0.7^{a}	40.8	0.6^{b}	0.018**
ADG (kg/d)	0.1	0.0	0.1	0.0	0.2	0.0	0.1	0.0	0.759^{ns}

Column means within a fixed effect bearing different superscripts significantly differ (P<0.05). Chest girth (CG), withers height (WH), body length (BL), body condition score (BCS), body weight (BWT), and average daily weight gain (ADG). Level of significance: ns not significant (P>0.05), * significant (P<0.05), ** highly significant (P<0.01), and *** very highly significant (P<0.001).

Spirulina and tissue fatty acids

The fatty acid composition of Spirulina analysed by GC-MS is listed in Table 6.1. The Spirulina diet had a significant effect on subcutaneous adipose, muscle, heart, kidney, and liver tissue FA compositions of 14:0, 15:0, 16:1n-7c, 16:0, 17:0, 18:2n-6, 18:1n-9, 18:1n-7c, 20:5n-3, 20:3n-6, 20:4n-3, 20:2n-6, 20:0, 22:5n-6, 22:6n-3, ∑SFA, and ∑n-6. In addition, the Spirulina diet significantly affected the profile of 16:0, 18:1n-9, 20:5n-3, and 22:5n-6 in two or more tissues (Table 6.3).

Table 6.3 Level of significance (*P* values) of subcutaneous adipose (A), heart (H), kidney (K), liver (L), and muscle (M) tissue fatty acid composition as percentage of total fatty acid (% total FA) as affected by *Spirulina* supplementations.

Fatty acid profile	A	M	H	K	L
14:0 Myristic acid	0.760 ns	0.252 ns	0.177 ns	0.900 ns	0.001
15:0 Pentadecanoic acid	0.661 ns	0.969 ns	0.045	0.453 ns	0.471 ns
16:1n-9c Palmitoleic acid	0.640 ns	0.575 ns	0.313 ns	0.616 ns	0.592 ns
16:1n-7c	0.043	0.651 ns	0.271 ns	0.510 ns	0.241 ns
16:0 Palmitic acid	0.021 ns	0.014	0.047	0.441 ns	0.326 ns
17:0 Heptadecanoic acid, or margaric acid	0.369 ns	0.004	0.060 ns	0.241 ns	0.106 ns
18:2n-6 Linoleic acid	0.027	0.665 ns	0.899 ns	0.233 ns	0.972 ns
18:3n-3 α-Linolenic acid	0.879 ns	0.474 ns	0.185 ns	0.241 ns	0.587 ns
18:1n-9 Oleic acid	0.441 ns	0.619 ns	0.047	0.041	0.289 ns
18:1n-7c Vaccenic acid	0.424 ns	0.029	0.536 ns	0.612 ns	0.863 ns
18:1n-7t	0.140 ns	0.702 ns	0.644 ns	0.628 ns	0.139 ns
18:0	0.870 ns	0.649 ns	0.575 ns	0.141 ns	0.904 ns
20:4n-6	0.398 ns	0.860 ns	0.582 ns	0.792 ns	0.571 ns
20:5n-3 Eicosapentaenoic acid	0.048	0.004	0.614 ns	0.475 ns	0.048
20:3n-6 Dihomo-γ-linolenic acid (DGLA)	0.722 ns	0.722 ns	0.223 ns	0.192 ns	0.036
20:4n-3 Eicosatetraenoic acid	.	.	0.040	0.705 ns	.
20:2n-6 Eicosadienoic acid	.	.	0.006	0.892 ns	.
20:0 Arachidic acid	0.003	0.597 ns	0.357 ns	0.953 ns	0.757 ns
22:5n-6 Docosapentaenoic acid	0.341 ns	0.531 ns	0.307 ns	0.011	0.010
22:6n-3 Docosahexaenoic acid (DHA)	0.951 ns	0.914 ns	0.552 ns	0.004	0.430 ns
22:5n-3 DPA-3	.	.	0.935 ns	0.267 ns	0.090 ns
22:0	.	.	0.259 ns	0.556 ns	0.811 ns
23:0	.	0.419 ns	.	0.919 ns	0.791 ns
24:0	.	0.795 ns	0.584 ns	0.605 ns	0.844 ns
∑SFA	0.414 ns	0.396 ns	0.713 ns	0.035	0.487 ns
∑MUFA	0.296 ns	0.419 ns	0.372 ns	0.310 ns	0.405 ns

∑PUFA	0.373 ns	0.812 ns	0.441 ns	0.677 ns	0.237 ns
∑n-3	0.933 ns	0.462 ns	0.331 ns	0.321 ns	0.107 ns
∑n-6	0.040	0.811 ns	0.774 ns	0.512 ns	0.784 ns

Level of significance: ns not significant (P>0.05), * significant (P<0.05), ** highly significant (P<0.01), and *** very highly significant (P<0.001).

a % lipid is the percentage lipid in raw tissue; ∑SFA is the sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0; ∑MUFA is the sum of 14:1n-5, 15:1n-6, 16:1n-9, 16:1n-7, Br17:1, 17:1n-8+a17:0, 17:1, 18:1n-9, 18:1n-7, 18:1n-5, 18:1, 19:1, 20:1n-11, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9, 22:1n-11, 22:1n-7, 24:1n-11, 24:1n-9, 24:1n-7; ∑PUFA is the sum of 16:3+16:4, 16:2, 18:4n-3, 18:3n-6, 18:2n-6, 18:3n-3, 20:4n-3, 20:4n-6, 20:5n-3, 20:3n-6, 20:2n-6, 21:5n-3, 22:6n-3, 22:5n-3, 22:5n-6, 22:4n-6, 24:6n-3, 24:5n-3; ∑n-3 PUFA is the sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 21:5n-3, 22:6n-3, 22:5n-3, 24:6n-3, 24:5n-3; ∑n-6 PUFA is the sum of 18:2n-6, 18:3n-6, 20:4n-6, 20:3n-6, 20:2n-6, 22:5n-6, 22:4n-6.

Table 6.4 Mean percentage composition of total fatty acids (% total fat), standard error (SEM), number of tissue samples (*n*), and level of significance (*P* value) of subcutaneous adipose tissues from Australian dual-purpose lambs[a,b,c]

Spirulina (% total FA)	Control		Low		Medium		High	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	3.0	0.2	3.1	0.1	2.2	0.4	2.6	0.4
15:0	0.7	0.0	0.6	0.1	0.6	0.1	0.6	0.1
16:1n-9c	0.4	0.0	0.3	0.0	0.3	0.0	0.4	0.0
16:1n-7c	0.9	0.2^{B}	1.1	0.0^{B}	1.2	0.0^{A}	1.2	0.0^{A}
16:0	24.1	0.1	25.4	0.0	22.7	0.1	24.3	0.1
17:0	1.9	0.1	2.1	0.1	1.8	0.1	2.0	0.1
18:2n-6	1.6	0.0^{B}	1.6	0.0^{B}	1.8	0.1^{A}	1.5	0.1^{B}
18:3n-3	1.5	0.1	1.4	0.2	1.4	0.1	1.4	0.1
18:1n-9	32.4	1.6	29.4	2.4	34.7	2.0	32.7	1.3
18:1n-7c	1.3	0.1	1.1	0.0	1.3	0.1	1.3	0.0
18:1n-7t	4.0	0.3	2.7	0.2	3.6	0.3	3.6	0.2
18:0	23.3	1.4	26.9	1.2	24.4	3.3	23.1	1.4
20:4n-6	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
20:5n-3	0.0	0.0^{B}	0.0	0.0^{B}	0.2	0.0^{A}	0.1	$0.^{1AB}$
20:2n-6	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
20:0	0.1	0.0^{B}	0.2	0.0^{A}	0.0	0.0^{c}	0.1	0.0^{B}
22:5n-6	0.1	0.0	0.2	0.1	0.0	0.0	0.1	0.1
22:6n-3	0.0	0.0	0.0	0.0	0.0	0.0	0.1	0.1
∑SFA	59.0	0.2	53.8	0.1	52.5	0.5	53.7	0.8
∑MUFA	44.2	0.6	42.6	0.4	37.1	0.4	42.6	0.4
∑PUFA	3.3	0.1	3.6	0.1	3.9	0.2	3.7	0.1
∑n-3	1.6	0.1	1.5	0.1	1.4	0.1	1.6	0.2
∑n-6	1.6	0.0^{C}	1.7	0.2^{B}	2.1	0.1^{A}	1.8^{B}	0.1^{BA}

[1] Means with different superscripts [A, B, C, D, E] within rows significantly differ (*P*<0.05).

[2] ∑SFA is the sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0; ∑MUFA is the sum of 14:1n-5, 15:1n-6, 16:1n-9, 16:1n-7, Br17:1, 17:1n-8+a17:0, 17:1, 18:1n-9, 18:1n-7, 18:1n-5, 18:1, 19:1, 20:1n-11, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9, 22:1n-11, 22:1n-7, 24:1n-11, 24:1n-9, 24:1n-7; ∑PUFA is the sum of 16:3+16:4, 16:2, 18:4n-3, 18:3n-6, 18:2n-6, 18:3n-3, 20:4n-3, 20:4n-6, 20:5n-3, 20:3n-6, 20:2n-6, 21:5n-3, 22:6n-3, 22:5n-3, 22:5n-6, 22:4n-6, 24:6n-3, 24:5n-3; ∑n-3 PUFA is the sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 21:5n-3, 22:6n-3, 22:5n-3, 24:6n-3, 24:5n-3; ∑n-6 PUFA is the sum of 18:2n-6, 18:3n-6, 20:4n-6, 20:3n-6, 20:2n-6, 22:5n-6, 22:4n-6.

[c] FA not found (%total FA = 0) were 20:3n-6, 20:4n-3, 22:5n-3, 22:0, 23:0, 24:0.

Tissue fatty acid content

Adipose:

In the subcutaneous adipose tissue, the level of 16:1n-7c composition was found to be higher by 1.2% in the tissues of animals that received medium and high levels of *Spirulina* compared to the control (0.9%) and low (1.1%) levels of supplementation (Table 6.4). The highest composition of 18:2n-6 (linoleic acid) was discovered in the tissues associated with the medium level of *Spirulina* supplementation (1.8%) with a significant difference ($P<0.05$), in contrast to the compositions for the other supplementation levels, which did not differ significantly (Table 6.4).

For 20:5n-3 (eicosapentaenoic acid), the highest composition of 0.2 (Table 6.4) was observed for the medium supplementation levels, which significantly differed ($P<0.05$) from the low and control supplementation levels. However, no significant difference was found compared to the high supplementation level (Table 6.4). In the adipose tissue, the highest level of 20:0 composition (arachidic acid) was 0.2% (Table 6.4), which was found in tissues associated with low level of supplementation with a significant difference ($P<0.01$). This was followed by 0.1% for the control and high supplementation levels (Table 6.4). The lowest level of arachidic acid in adipose tissues was recorded for the medium level of supplementation. The medium level of supplementation produced the highest ∑n-6 composition of 2.1 (Table 6.4), which significantly differed from other tissues. The ∑n-6 composition was observed to be significantly higher for both low and high supplementations compared to the control group (Table 6.4).

Muscle*:*

The level of palmitic acid (16:0) in the muscle tissue of the control group was higher than that for the low, medium, and high levels of supplementation with a significant difference ($P<0.05$) (Table 6.5).

Table 6.5 Mean percentage composition of total fatty acids (% total fat), standard error (SEM), number of tissue samples (*n*), and level of significance (*P* value) of muscle tissues from Australian dual-purpose lambs[a,b,c]

Spirulina (% total FA)	Control		Low		Medium		High	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	1.8	0.2	1.1	0.7	1.8	0.2	1.9	0.2
15:0	0.5	0.0	0.4	0.1	0.5	0.1	0.4	0.0
16:1n-9c	0.3	0.0	0.3	0.0	0.3	0.0	0.2	0.0
16:1n-7c	1.1	0.1	1.1	0.3	1.1	0.1	1.3	0.1
16:0	23.8	0.1^{A}	22.2	0.1^{B}	22.0	0.0^{B}	22.6	0.2^{B}
17:0	1.4	0.0^{B}	1.6	0.1^{A}	1.3	0.0^{B}	1.3	0.1^{B}
18:2n-6	4.5	0.6	3.8	0.6	3.7	0.3	4.1	0.4
18:3n-3	2.0	0.1	1.7	0.2	2.0	0.1	2.0	0.1
18:1n-9	35.5	1.0	36.4	1.2	36.5	0.7	35.5	1.5
18:1n-7c	1.4	0.1^{B}	1.5	0.1^{A}	1.5	0.0^{AB}	1.5	0.1^{A}
18:1n-7t	3.1	0.2	2.7	0.1	2.8	0.1	2.8	0.2
18:0	20.1	0.7	20.6	1.2	19.2	0.8	20.0	0.7
20:4n-6	0.7	0.2	0.5	0.0	0.7	0.1	0.7	0.1
20:5n-3	0.1	0.1^{B}	0.2	0.1^{B}	0.5	0.1^{A}	0.5	0.1^{A}
20:3n-6	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
20:0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
22:5n-6	0.1	0.0	0.1	0.0	0.1	0.1	0.0	0.0
22:6n-3	0.1	0.1	0.1	0.0	0.1	0.0	0.1	0.0
22:5n-3	0.2	0.1	0.2	0.0	0.3	0.1	0.4	0.1
∑SFA	46.6	1.2	48.1	1.0	45.7	1.0	47.1	1.3
∑MUFA	44.8	1.0	45.1	1.1	46.4	1.0	44.7	1.3
∑PUFA	8.6	1.1	6.9	0.7	7.9	0.6	8.3	0.8
∑n-3	2.9	0.3	2.1	0.2	3.0	0.2	3.0	0.3
∑n-6	5.5	0.8	4.5	0.6	4.7	0.4	5.0	0.6

[a] Means with different superscripts [A, B, C, D, E] within rows significantly differ ($P<0.05$).
[b] ∑SFA is the sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0; ∑MUFA is the sum of 14:1n-5, 15:1n-6, 16:1n-9, 16:1n-7, Br17:1, 17:1n-8+a17:0, 17:1, 18:1n-9, 18:1n-7, 18:1n-5, 18:1, 19:1, 20:1n-11, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9, 22:1n-11, 22:1n-7, 24:1n-11, 24:1n-9, 24:1n-7; ∑PUFA is the sum of 16:3+16:4, 16:2, 18:4n-3, 18:3n-6, 18:2n-6, 18:3n-3, 20:4n-3, 20:4n-6, 20:5n-3, 20:3n-6, 20:2n-6, 21:5n-3, 22:6n-3, 22:5n-3, 22:5n-6, 22:4n-6, 24:6n-3, 24:5n-3; ∑n-3 PUFA is the sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 21:5n-3, 22:6n-3, 22:5n-3, 24:6n-3, 24:5n-3; ∑n-6 PUFA is the sum of 18:2n-6, 18:3n-6, 20:4n-6, 20:3n-6, 20:2n-6, 22:5n-6, 22:4n-6.
[c] FA not found (%total FA = 0) were 20:4n-3, 20:2n-6, 22:0, 23:0, 24:0.

For 17:0 (heptadecanoic acid) muscle composition, the low level of supplementation differed significantly ($P<0.01$) from other supplementation levels, having the highest composition of 1.6% (Table 6.5). Both the low and high levels of supplementation had the highest 18:1n-7c muscle composition (1.5%), which significantly differed ($P<0.05$) from the control and medium levels (Table 6.5). For 20:5n-3 (eicosapentaenoic acid), the highest muscle composition of 0.5% was found with the medium and high

supplementation levels, which significantly differed (P<0.05) from the control and low-level supplementation groups (Table 6.5).

Heart:
The lowest 15:0 (pentadecanoic acid) fatty acid composition in heart tissue of 0.2% was discovered in tissues supplemented with low levels, while the compositions for other supplementations did not differ significantly (Table 6.6).

Table 6.6 Mean percentage composition of total fatty acids (% total fat), standard error (SEM), number of tissue samples (*n*), and level of significance (*P* value) of heart tissues from Australian dual-purpose lambs[a,b]

Spirulina (% total FA)	Control		Low		Medium		High	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	0.7	0.2^{B}	1.2	0.4^{A}	0.8	0.2^{BA}	0.5	0.1^{B}
15:0	0.4	0.0^{A}	0.2	0.0^{B}	0.0	0.0^{B}	0.4	0.0^{A}
16:1n-9c	0.2	0.0	0.2	0.0	0.3	0.0	0.2	0.0
16:1n-7c	0.4	0.1	0.1	0.1	0.4	0.1	0.3	0.1
16:0	15.1	0.1^{A}	14.7	0.6^{AB}	14.1	0.1^{B}	15.0	0.4^{A}
17:0	1.3	0.0	1.5	0.1	1.3	0.0	1.4	0.0
18:2n-6	17.1	1.2	15.5	1.6	16.5	1.5	15.7	1.4
18:3n-3	3.6	0.4	2.1	0.1	3.4	0.3	3.0	0.2
18:1n-9	18.1	0.4^{B}	19.9	0.2^{A}	19.8	0.1^{A}	19.0	0.1^{AB}
18:1n-7c	1.9	0.1	2.0	0.2	2.0	0.1	1.9	0.1
18:1n-7t	2.1	0.2	1.7	0.2	2.3	0.2	2.2	0.2
18:0	19.3	1.0	21.0	1.8	21.5	1.4	21.6	1.3
20:4n-6	5.6	0.5	4.6	0.8	4.1	0.6	4.8	0.7
20:5n-3	3.1	0.6	1.5	0.2	2.3	0.2	2.6	0.6
20:3n-6	0.6	0.0	0.5	0.1	0.5	0.1	0.6	0.0
20:4n-3	0.1	0.0^{B}	0.2	0.0^{A}	0.1	0.0^{B}	0.1	0.0^{B}
20:2n-6	0.0	0.0^{B}	0.0	0.0^{B}	0.1	0.0^{A}	0.0	0.0^{B}
20:0	0.1	0.0^{C}	0.3	0.0^{A}	0.1	0.0^{C}	0.2	0.0^{B}
22:5n-6	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
22:6n-3	1.3	0.2	0.9	0.2	0.7	0.1	1.0	0.2
22:5n-3	1.9	0.3	1.6	0.3	1.2	0.2	1.5	0.2
22:0	0.3	0.1	0.2	0.0	0.1	0.0	0.3	0.1
23:0	0.2	0.1	0.3	0.0	0.1	0.0	0.3	0.0
24:0	0.2	0.0	0.2	0.0	0.1	0.0	0.2	0.0
∑SFA	37.8	1.2	41.1	3.3	40.1	1.8	40.7	1.5
∑MUFA	28.2	0.9	30.9	1.4	30.3	0.9	29.2	1.1
∑PUFA	34.0	1.4	27.9	3.3	29.6	2.5	30.1	2.0
∑n-3	10.0	1.0	6.3	0.8	7.8	0.6	8.3	0.9
∑n-6	23.4	1.3	21.0	2.5	21.4	2.0	21.4	1.7

[a] Means with different superscripts [A, B, C, D, E] within rows significantly differ (P<0.05).
[b] ∑SFA is the sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0; ∑MUFA is the sum of 14:1n-5, 15:1n-6, 16:1n-9, 16:1n-7, Br17:1, 17:1n-8+a17:0, 17:1, 18:1n-9, 18:1n-7, 18:1n-5, 18:1, 19:1, 20:1n-11, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9, 22:1n-11, 22:1n-7, 24:1n-11, 24:1n-9, 24:1n-7; ∑PUFA is the sum of 16:3+16:4, 16:2, 18:4n-3, 18:3n-6, 18:2n-6, 18:3n-3, 20:4n-3, 20:4n-6, 20:5n-3, 20:3n-6, 20:2n-6, 21:5n-3, 22:6n-3, 22:5n-3, 22:5n-6, 22:4n-6, 24:6n-3, 24:5n-3; ∑n-3 PUFA is the sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 21:5n-3, 22:6n-

3, 22:5n-3, 24:6n-3, 24:5n-3; ∑n-6 PUFA is the sum of 18:2n-6, 18:3n-6, 20:4n-6, 20:3n-6, 20:2n-6, 22:5n-6, 22:4n-6.

The 16:0 (palmitic acid) fatty acid composition differed (P<0.05) in heart tissues supplemented with low and medium levels compared to the control (Table 6.6). The low level of supplementation was associated with the highest composition of 19.9%, followed by the medium level (19.8%) (Table 6.6). Tissues supplemented with medium levels had the highest 20:2n-6 (eicosadienoic acid) composition of 0.1%, which differed significantly (P<0.01), whereas other supplementation compositions did not differ significantly (Table 6.6). The 18:1n-9 (oleic acid) heart composition was discovered to be higher for both the low and medium supplementation levels (Table 6.6). The 20:4n-3 (eicosatetraenoic acid) composition in heart tissue was found to be highest at 0.1% and significantly differed from the control, low, and medium levels of supplementations (Table6. 6).

Kidney:

The 18:1n-9 (oleic acid) composition of the kidney was higher for the low-supplementation group compared to the control, medium, and high-supplementation groups (P<0.05) (Table 6.7).

The 22:5n-6 (docosapentaenoic acid) composition of the kidney for the medium and high supplementation was highest at 0.9% and 0.5%, respectively, which significantly differed (P<0.05), whereas the other compositions did not (Table 6.7). The 22:6n-3 (docosahexaenoic acid) composition of the kidney for the high supplementation was the highest at 3.4% with a significant difference (P<0.05) from the control, low, and medium supplementation levels (Table 6.7). The ∑PUFA kidney composition of the medium and high supplementation levels was higher compared to the control and low levels of supplementation (P<0.05) (Table 6.7).

Table 6.7 Mean percentage composition of total fatty acids (% total fat), standard error (SEM), number of tissue samples (*n*), and level of significance (*P* value) of kidney tissues from Australian dual-purpose lambs[a,b]

Spirulina (% total FA)	Control		Low		Medium		High	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	0.2	0.1	0.2	0.2	0.1	0.1	0.2	0.1
15:0	0.3	0.0	0.2	0.1	0.3	0.1	0.3	0.0
16:1n-9c	0.2	0.0	0.1	0.1	0.2	0.0	0.2	0.1
16:1n-7c	0.3	0.0	0.3	0.1	0.4	0.1	0.3	0.1
16:0	19.2	0.8	19.0	1.3	19.3	1.7	18.2	0.3
17:0	1.4	0.1	1.6	0.1	1.3	0.1	1.3	0.1
18:2n-6	8.9	0.4	10.7	0.6	9.0	0.6	9.7	0.4
18:3n-3	1.9	0.3	1.6	0.4	4.4	2.3	2.1	0.3
18:1n-9	17.1	0.3^{A}	15.8	0.4^{B}	15.2	0.2^{B}	15.2	1.0^{B}
18:1n-7c	1.4	0.1	1.8	0.1	1.5	0.2	1.4	0.1
18:1n-7t	1.6	0.2	0.9	0.1	1.3	0.3	1.2	0.2
18:0	22.5	1.0	21.1	1.1	19.0	1.6	19.8	1.2
20:4n-6	7.9	1.0	9.4	1.5	8.4	1.3	10.3	1.3
20:5n-3	5.0	0.9	2.7	0.7	5.1	0.9	5.5	0.7
20:3n-6	0.6	0.1	0.7	0.1	0.5	0.1	0.7	0.1
20:4n-3	0.0	0.0	0.1	0.0	0.0	0.0	0.0	0.0
20:2n-6	0.1	0.0	0.1	0.0	0.1	0.0	0.0	0.0
20:0	0.2	0.0	0.1	0.0	0.2	0.0	0.2	0.0
22:5n-6	0.1	0.1^{B}	0.0	0.1^{B}	0.9	0.0^{A}	0.5	0.1^{A}
22:6n-3	2.5^{B}	0.2	2.7	0.1^{B}	2.8	0.2^{A}	3.4	0.1^{A}
22:5n-3	2.7	0.3	2.6	0.3	2.6	0.4	3.2	0.2
22:0	1.0	0.1	0.9	0.2	1.5	0.3	1.0	0.1
23:0	0.2	0.1	0.2	0.1	0.3	0.1	0.2	0.1
24:0	0.8	0.1	0.9	0.3	1.3	0.4	0.7	0.1
∑SFA	46.6	1.6	44.7	2.3	44.4	0.9	42.7	1.3
∑MUFA	22.7	0.7	23.5	1.8	22.6	1.6	21.9	1.0
∑PUFA	30.8	0.5^{B}	31.8	0.1^{B}	33.9	0.5^{A}	35.4	0.6^{A}
∑n-3	12.5	1.5	9.8	0.7	14.7	1.8	14.0	0.9
∑n-6	18.0	1.2	21.8	1.2	18.1	1.6	21.0	1.5

[a] Means with different superscripts [A, B, C, D, E] within rows significantly differ ($P<0.05$).

[b] ∑SFA is the sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0; ∑MUFA is the sum of 14:1n-5, 15:1n-6, 16:1n-9, 16:1n-7, Br17:1, 17:1n-8+a17:0, 17:1, 18:1n-9, 18:1n-7, 18:1n-5, 18:1, 19:1, 20:1n-11, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9, 22:1n-11, 22:1n-7, 24:1n-11, 24:1n-9, 24:1n-7; ∑PUFA is the sum of 16:3+16:4, 16:2, 18:4n-3, 18:3n-6, 18:2n-6, 18:3n-3, 20:4n-3, 20:4n-6, 20:5n-3, 20:3n-6, 20:2n-6, 21:5n-3, 22:6n-3, 22:5n-3, 22:5n-6, 22:4n-6, 24:6n-3, 24:5n-3; ∑n-3 PUFA is the sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 21:5n-3, 22:6n-3, 22:5n-3, 24:6n-3, 24:5n-3; ∑n-6 PUFA is the sum of 18:2n-6, 18:3n-6, 20:4n-6, 20:3n-6, 20:2n-6, 22:5n-6, 22:4n-6.

Liver:

The liver from the control and low-supplementation groups had the highest 14:0 (myristic acid) compositions of 0.6% and 0.5%, respectively, with significance ($P<0.01$), followed by the lowest observed 14:0 composition of 0.3% in the medium and high-supplementation groups (Table 6.8).

Table 6.8 Mean percentage composition of total fatty acids (% total fat), standard error (SEM), number of tissue samples (*n*), and level of significance (*P* value) of liver tissues from Australian dual-purpose lambs[a,b,c]

Spirulina (% total FA)	Control		Low		Medium		High	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
14:0	0.6	0.2^{A}	0.5	0.2^{A}	0.3	0.1^{B}	0.3	0.1^{B}
15:0	0.5	0.1	0.5	0.1	0.5	0.0	0.4	0.0
16:1n-9c	0.4	0.1	0.4	0.1	0.4	0.0	0.4	0.0
16:1n-7c	0.6	0.1	0.4	0.1	0.7	0.1	0.5	0.1
16:0	19.8	1.4	22.0	1.0	18.9	0.9	19.7	1.2
17:0	1.5	0.1	2.0	0.1	1.3	0.1	1.5	0.1
18:2n-6	6.4	0.5	6.2	0.7	6.4	0.4	6.4	0.5
18:3n-3	3.0	0.2	2.4	0.7	3.4	0.3	3.0	0.3
18:1n-9	21.0	1.1	25.6	1.1	22.0	1.0^{B}	22.1	1.0^{B}
18:1n-7c	1.3	0.1	1.3	0.1	1.3	0.0	1.2	0.0
18:1n-7t	2.4	0.4	2.0	0.1	1.8	0.3	2.4	0.3
18:0	22.5	2.6	23.9	1.8	21.9	1.4	23.3	1.3
20:4n-6	4.4	0.9	2.9	0.8	4.4	0.6	3.8	0.7
20:5n-3	1.0	0.6^{B}	3.1	0.4^{A}	3.5	0.5^{A}	2.8	0.6^{A}
20:3n-6	0.3	0.1^{B}	0.5	0.1^{A}	0.6	0.1^{A}	0.5	0.1^{A}
20:0	0.1	0.0	0.1	0.0	0.1	0.0	0.1	0.0
22:5n-6	0.1	0.0^{B}	0.1	0.9^{B}	1.1	0.0^{A}	0.1	0.0^{B}
22:6n-3	3.9	0.7	1.9	0.3	3.7	0.3	3.0	0.5
22:5n-3	3.3	0.5	1.6	0.4	3.8	0.4	3.3	0.5
22:0	0.2	0.1	0.1	0.0	0.2	0.1	0.2	0.1
23:0	0.1	0.1	0.2	0.1	0.2	0.1	0.2	0.1
24:0	0.2	0.1	0.1	0.0	0.2	0.1	0.2	0.1
∑SFA	46.1	2.2	50.0	2.5	44.4	1.2	46.7	1.7
∑MUFA	28.6	1.3	32.4	1.3	29.0	1.1	29.8	1.1
∑PUFA	25.3	2.7	17.6	2.4	26.6	1.6	23.5	2.3
∑n-3	13.4	1.7	6.9	1.5	14.6	1.1^{A}	12.3	1.5
∑n-6	11.6	1.3	10.5	1.0	11.7	1.0	11.0	1.2

[a] Means with different superscripts $^{A, B, C, D, E}$ within rows significantly differ ($P<0.05$).

[b] ∑SFA is the sum of 14:0, 15:0, 16:0, 17:0, 18:0, 20:0, 21:0, 22:0, 23:0, 24:0; ∑MUFA is the sum of 14:1n-5, 15:1n-6, 16:1n-9, 16:1n-7, Br17:1, 17:1n-8+a17:0, 17:1, 18:1n-9, 18:1n-7, 18:1n-5, 18:1, 19:1, 20:1n-11, 20:1n-9, 20:1n-7, 20:1n-5, 22:1n-9, 22:1n-11, 22:1n-7, 24:1n-11, 24:1n-9, 24:1n-7; ∑PUFA is the sum of 16:3+16:4, 16:2, 18:4n-3, 18:3n-6, 18:2n-6, 18:3n-3, 20:4n-3, 20:4n-6, 20:5n-3, 20:3n-6, 20:2n-6, 21:5n-3, 22:6n-3, 22:5n-3, 22:5n-6, 22:4n-6, 24:6n-3, 24:5n-3; ∑n-3 PUFA is the sum of 18:3n-3, 18:4n-3, 20:4n-3, 20:5n-3, 21:5n-3, 22:6n-3, 22:5n-3, 24:6n-3, 24:5n-3; ∑n-6 PUFA is the sum of 18:2n-6, 18:3n-6, 20:4n-6, 20:3n-6, 20:2n-6, 22:5n-6, 22:4n-6.

[c] FA not found (%total FA = 0) were 20:4n-3, 20:2n-6.

The 20:5n-3 (eicosapentaenoic acid) composition of the liver from the low, medium, and high-supplementation groups were significantly higher ($P<0.05$) than the control supplement level of 1.0% (Table 6.8). For 20:3n-6 (dihomo-γ-linolenic acid) in the liver, the highest compositions were found for both the medium and high supplementation levels at 0.6% and 0.5%, respectively (Table 6.8). The composition of 22:5n-6 (docosapentaenoic acid) in the liver was the highest at 1.1% for the medium-supplementation group, which significantly differed from the other supplementation levels ($P<0.05$) (Table 6.8).

Discussion

In this study, FA data were converted into percentages of total FA composition (as % total FA). Aspects of the FA profile were evaluated to provide deeper insight into the effect of *Spirulina* supplementation on FA composition in Australian dual-purpose lambs. It was demonstrated that the medium-level *Spirulina* diet resulted in higher polyunsaturated fatty acid (PUFA) compositions.

Studies have demonstrated that diets with high FA content have a major impact on the fatty acid composition of ruminants (Alfaia et al., 2009, Raes et al., 2004, Wachira et al., 2007). Research has shown that ruminant n-3, n-6, and PUFA content can be improved by increased dietary intake of α-linolenic acid (ALA) and linoleic acid (LA) (Wachira et al., 2007, Wood and Enser, 1997, Wood et al., 2004). This stems from the use of ALA as a precursor to n-3 PUFA, including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (Abeywardena and Patten, 2011, Belay et al., 1993, Daley et al., 2010, Kouba and Mourot, 2011, Raes et al., 2004). Similarly, LA is a precursor to n-6 PUFAs, including its conversion to gamma-linolenic acid (GLA), which is converted to dihomo-gamma-linolenic acid (DGLA), and which in turn is converted to arachidonic acid (ARA) (Doreau et al., 2010, Price et al., 2000, Woods and Fearon, 2009).

The *Spirulina* diet was rich in the essential fatty acids ALA, LA, and DGLA (Iwata et al., 1990, Qureshi et al., 1996, Ross and Dominy, 1990). Thus, it is acceptable to presume that these dietary ingredients contributed to the observed levels of individual and total tissue n-3 and n-6 PUFAs (Fokkema et al., 2002, Pethick et al., 2010). In our study, the medium-level *Spirulina* diet increased eicosapentaenoic acid and linoleic acid significantly in subcutaneous adipose tissue, which increased the percentage of n-6 PUFA. Research has demonstrated that eicosapentaenoic acid is an essential and physiologically significant n-3 fatty acid (Abeywardena and Patten, 2011, Belay et al., 1993, Daley et al., 2010, Mapiye et al., 2011, Woods and Fearon, 2009).

It was discovered that the medium *Spirulina* diet increased eicosapentaenoic acid in subcutaneous adipose, muscle, and liver tissues. EPA is one of the types of n-3 needed by the human body, which cannot make EPA fatty acid and must thus acquire it from food or supplement sources. The low *Spirulina* diet reduced the percentage of pentadecanoic acid in heart tissue (Hoashi et al., 2008, Scollan et al., 2001). This is because the *Spirulina* supplement is rich in α-Linolenic acid.

Pentadecanoic acid is a saturated FA found in milk fat from cows (Cooper et al., 2004, DeBusk, 2010), and it is also detected in hydrogenated mutton fat (Scollan et al., 2001, Woods and Fearon, 2009). This finding suggests that the low-level *Spirulina* diet reduces the conversion of unsaturated fatty acids to saturated fatty acids by the biohydrogenation process (Kouba and Mourot, 2011, Pethick et al., 2010) in crossbred lambs. An increase in eicosadienoic acid (20:2n-6) was observed in the tissues associated with the low-level *Spirulina* diet.

Eicosadienoic acid (EDA) is a naturally occurring n-6 PUFA found mainly in animal tissues (Daley *et al.*, 2010). EDA is elongated from linoleic acid (LA) (Kouba and Mourot, 2011, Price et al., 2000) and can be further metabolised to dihomo-γ-linolenic acid (DGLA) and arachidonic acid (AA) (Daley et al.,

2010, Mapiye et al., 2011, Price et al., 2000). This indicates that an LA-rich diet can increase EDA elongation, which is then further metabolized to arachidonic acid (Mapiye et al., 2011, Moibi and Christopherson, 2001, Nguyen et al., 2010). The role of arachidonic acid includes keeping cell membranes flexible and permeable (Pethick et al., 2010, Price et al., 2000), and it also promotes muscle growth (Fokkema et al., 2002, Rowe, 2010, Santos-Silva et al., 2002).

It was demonstrated that the medium *Spirulina* diet significantly affected the percentage of docosapentaenoic acid in kidney tissue. Docosapentaenoic acid is an n-6 FA (Kouba and Mourot, 2011). It is formed metabolically from LA and is a constituent of animal glycerophospholipids (Scollan et al., 2001, Smet et al., 2004, Wachira et al., 2007). This highlights that a *Spirulina* diet rich in LA increases other n-6 PUFAs in Australian crossbred lambs, all the way through 22:5n-6.

The *Spirulina* diets were shown to affect the composition of oleic acid in *longissimus dorsi* muscle tissue. Oleic acid is classified as a monounsaturated *n*-9 FA (Daley et al., 2010, Kouba and Mourot, 2011). Previous studies have found significant variation of FA composition in response to dietary supplementation (Alfaia et al., 2009, Doreau et al., 2010, Mapiye et al., 2011, Scollan et al., 2001). The biosynthesis of oleic acid involves the action of the enzyme stearoyl-CoA 9-desaturase (Wood *et al.*, 2008) acting on stearoyl-CoA (Price et al., 2000, Woods and Fearon, 2009) and the dehydrogenation of stearic acid to give the MUFA derivative oleic acid (Wood et al., 2008, Wood et al., 2004, Woods and Fearon, 2009).

The Spirulina diet resulted in the reduction of 16:0 fatty acid, which is known as palmitic acid. This acid is the first produced during fatty acid synthesis (Wood et al., 2008, Wood et al., 2004). Palmitic acid is a precursor to longer fatty acids (Wachira et al., 2002). It negatively feeds back on acetyl-CoA carboxylase (ACC) (Kouba and Mourot, 2011, Santos-Silva et al., 2002), which is responsible for converting acetyl-CoA to malonyl-CoA (Smet et al., 2004, Wood and Enser, 1997), which in turn is used

to add to the growing acyl chain, thus preventing further palmitate generation (Price et al., 2000, Raes et al., 2004, Rowe, 2010). Research has demonstrated that palmitic acid increases the risk of developing cardiovascular diseases (Abeywardena and Patten, 2011, Belay et al., 1993, Fokkema et al., 2002). In this study, the tissue and composition of lamb muscle and heart were observed to respond to the medium Spirulina diet supplementation, which resulted in the lowest levels of palmitic acid and highest levels of linoleic acid and n-6 PUFA.

Conclusions

This study has identified the composition variation in the FA profiles of lamb adipose, muscle, heart, kidney, and liver tissues attributable to differences in the level of *Spirulina* supplementation. The medium level of *Spirulina* supplementation increased the FA composition in some tissues and elevated the proportion of n-3 and n-6 PUFAs in all tissues and organs. Low and high levels of *Spirulina* supplementation slightly increased PUFAs in some tissues and organs, but excessive supplementation can suppress optimal sheep growth because the excess protein is deaminated and lost in the urine or broken down in the liver, leading to fatty liver and ketosis. We suggest the use of 10% *Spirulina* supplementation in order to increase lamb production with higher PUFA composition.

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Chapter 7

Single Nucleotide Polymorphisms of the Ovine Adrenergic beta-3 receptor Gene in Crossbred Australian Sheep Supplemented with *Spirulina* (*Arthrospira platensis*) Cyanobacterial Microalgae

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Abstract

The adrenergic-receptor beta3 (*ADRB3*) gene is an obesity gene that is involved in the regulation of energy balance and a variety of physiological functions by increasing lipolysis and thermogenesis. *Spirulina* (*Arthrospira platensis*) is a blue-green cyanobacterial alga containing 60-70% protein with an extensive history of human consumption and, more recently, inclusion in animal feeds. We had earlier demonstrated that low level oral supplementation by drenching prime lambs with 100 mL/head/day of 1g of *Spirulina* powder dissolved in 10 mL of water (10%wt:vol) increased live-weight and body conformation measurements in Black Suffolk (BS) x Merino

crossbred sheep. The hypothesis that genetics-nutrition interactions between sheep breeds with fewer mutations at the ADRB3 locus and an optimal Spirulina supplementation level will increase lean meat production was tested in the current study. Forty-eight crossbred Australian prime lambs sired by four rams of diverse breeds under the same pasture-based management conditions were subjected to a nine-week feeding trial with *Spirulina,* followed by genomic DNA extraction and single nucleotide polymorphism (SNP) analysis. Eleven SNPs in both the coding and non-coding regions of the ovine *ADRB3* gene were detected. Nine of the SNPs were in exon I and two were in intron. Variations in SNP frequencies were highly significant (P <0.0001) between all sheep breeds. The maximum and minimum number of SNPs were found in purebred Merinos (4.83) and Black Suffolk x Merino (BS) crossbreds (1.67). In total, one indel and six transverse mutations were detected that resulted in six amino acid substitutions. BS crossbreds had the lowest frequency of mutation and amino acid substitutions in their population in agreement with our hypothesis. In conclusion, BS sheep genetics matched with low levels (100 mL/head/day) of *Spirulina* supplementation can lead to higher meat production with less fat content in a typical pasture-based sheep production system.

Keywords: β_3-adrenergic receptor gene, *Spirulina (Arthrospira platensis)*, SNP polymorphisms, sheep

Introduction

Sheep are one of the most economically important species of domesticated livestock for producing milk, meat, skin, and wool for humans (Wu *et al.*, 2012; Zhang *et al.*, 2013). In meat production, saleable meat yield is a crucial determinant of the financial returns and economic value accruable to producers. It is estimated by the ratio of muscle to carcass weights (Gardner *et al.*, 2010). Fat content in the carcass is an essential element for the perception of texture, flavour, and juiciness.

It is also a source of essential fatty acids that cannot be synthesised by humans, but nowadays it is considered as an unpopular and unhealthy meat constituent by many consumers (Wood *et al.*, 2008). Hence, researchers have been keenly interested in exploring ways and means of manipulating the fatty acid composition of meat by altering the ratio of saturated (SFA) to unsaturated (USFA) fatty acids. Achievement of this feat seems possible with the use of molecular markers in animal breeding programmes in combination with strategic nutritional supplementation with nutrient-dense feeds. Therefore, existing knowledge gaps in genetics-nutrition interactions for optimal lamb performance and healthy meat production need to be filled. This study intends to contribute in this regard by utilising Australian prime lambs and a dual-purpose sheep production system.

The β_3-adrenergic receptor (*ADRB3*), also known as β_3-adrenoreceptor, is a G-protein-coupled receptor predominantly located in the adipose tissue (Hu *et al.*, 2010; Wu *et al.*, 2012). It plays a major role in regulating mammalian energy storage and expenditure under mediating effects of the sympathetic nervous system (Hu *et al.*, 2010; Wu *et al.*, 2011). It is also the main mediator of the lipolytic and thermogenetic effect of high catecholamine (in particular norepinephrine) concentration in brown and white adipose tissues in rodents (Wu *et al.*, 2011; Forrest *et al.*, 2007). The receptor's primary role is speculated to be to regulate the resting metabolic rate and lipolysis (Forrest *et al.*, 2003). In humans, polymorphisms in the *ADRB3* gene have been associated with diabetes and obesity, where tryptophan is replaced by arginine at position 64 (Try64Arg) (Hu *et al.*, 2010; Wu *et al.*, 2012). In obese rats, a decreased level of *ADRB3* expression has been found in brown and white adipose tissue (Hu *et al.*, 2010). In addition, mice with a disrupted *ADRB3* gene had a decrease in lean body mass and a modest increase in body fat (Hu *et al.*, 2010). In sheep, polymorphisms of the *ADRB3* gene have been associated with the economically important

quantitative traits of birth weight, growth rate, carcass composition, cold survival, wool staple strength, and wool yield (Forrest *et al.*, 2003; 2007; Hu *et al.*, 2010).

Spirulina (Arthrospira platensis), an edible blue-green microalga, is a human and animal nutritional supplement that contains about 60%-70% protein, all essential amino acids, carotenoids, vitamins, and minerals (Belay et al., 1993; Ciferri et al., 1983; Holman et al., 2012; Holman and Malau-Aduli, 2013). Kulpys et al. (2009) demonstrated that dairy cows supplemented with Spirulina had a 21% increase in milk production. Holman and Malau-Aduli (2013) have published a review of the importance, sustainability, and commercial production of Spirulina with the global locations of production companies in Australia and other countries around the world. In dual-purpose prime lamb production, profitability is driven primarily by protein-rich supplementation. As a result, a gain in higher lamb production with healthier meat composition is a subject of immense interest, particularly in the highly successful Australian sheep industry. However, to our current knowledge, there is no published investigation of polymorphisms of the ovine ADRB3 gene and its association with production traits utilising Spirulina supplementation strategies anywhere in the world. Therefore, our research objective was to detect polymorphisms of this gene in Australian crossbred sheep and to evaluate its association with Spirulina supplementation level with a view to potentially increase lamb production with low fat content.

Materials and methods

Animals, management and phenotypic data collection

All procedures involving animals were approved by the University of Tasmania Animal Ethics Committee, and were conducted in accordance with the 1993 Tasmanian Animal Welfare Act and the 2004 Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The experimental flock at the University of Tasmania Farm, Cambridge, Hobart, utilised for this study comprised of 48 weaned lambs from purebred Merino dams sired by White Suffolk, Black Suffolk,

Dorset, and Merino rams under the same management conditions. All animals were maintained on ryegrass pastures as the basal diet. At six weeks of age, they were balanced by sire breed and gender and randomly allocated into three treatment groups: the control group grazing without Spirulina (0%), and low (100 mL/head/day in the ratio of 1g of Spirulina powder:10 mL of water or 10% wt/vol) and high (200 mL/head/day in the ratio of 2g of Spirulina powder:10mL of water or 20% wt/vol) Spirulina supplementation levels. The Spirulina powder was purchased from a commercial producer in Darwin, Northern Territory, Australia (TAAU, NT, Australia). Lambs were daily supplemented according to their assigned Spirulina treatment before being released into paddocks for grazing. The supplementary feeding trial continued for nine weeks after an initial three weeks of adjustment. Live weight (LWT), body condition score (BCS), body length (BL), withers height (WH), chest girth (CG), and average daily gain (ADG) were recorded weekly over this period. Details of the procedures for recording these growth and body conformation measurements had previously been described (Holman et al., 2012). At the end of the feeding trial, blood samples were taken by jugular venipuncture before the animals were slaughtered at a commercial abattoir in Gretna, Tasmania, for carcass and sensory evaluation of meat quality.

Genomic DNA Extraction

Blood samples, after collection from the 48 weaners, were stored at -80 °C until ready for genomic DNA extraction. Genomic DNA was extracted from the samples in triplicates (total of 144 samples) using Ultraclean Tissue and Blood Spin DNA Isolation Kits (MoBio, Solana Beach, CA). The purity of the extracted DNA was quantified using the NanoDrop 8000 spectrophotometer (NanoDrop, Wilmington, DE).

Polymerase Chain Reaction (PCR) Primers, Interrogation Primer Design and PCR Amplification

To amplify a series of overlapping fragments of the ovine ADRB3 gene sequence (GeneBank Accession Number DQ269497) (Wu et al., 2012), seven pairs of PCR and interrogation primers were

designed using Primo SNP 3.4: SNP PCR Primer Design software (Chang Bioscience, 2004) Table 8.1.

Table 7.1 PCR primer sequences, fragment length, and annealing temperatures of interrogation primers used in SNP panel sequencing

Primer names	Primer sequences		Length (bp)	Annealing temperatures (Cº)
P1	Forward	5´-CTCATTCCTTCCCGCCCCACG-3´	305	64
	Reverse	5´-ACGACCAGAAGCCCCACCAC-3´		64
	Interrogation	5´- aaaaaaaaaaaTTTGGGTGCCACCGCTGGG-3´	30	64
P2	Forward	5´-AGGCAACCTGCTGGTAATC-3´	335	64
	Reverse	5´-GCGGACACGACCCAC-3´		64
	Interrogation	5´- aaaaaaaaaaaaaaaaaaaaaTCAGGCCGGGGGAATCGCT-3´	40	64
P3	Forward	5´-GCCTCCAACATGCCCTAC-3´	397	64
	Reverse	5´-CATAGCCCAGCCAGTTAAG-3´		64
	Interrogation	5´- aaaaaaaaaaaaaaaaaaaaaaaaaaaaaCCAGCTCACCCTGCTCAGC-3´	48	64
P4	Forward	5´-CCTCTGCGGGAACAC-3´	350	64
	Reverse	5´-CCCTTCGCCTCTATTACCT-3´		64
	Interrogation	5´- aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaATGAGGGGAAGGGTCT CCCA-3´	55	64
P5	Forward	5´-CCGCTCGACGGGTAGGTAA-3´	392	64
	Reverse	5´-GCTCGCGTGATGGTAAGAAT-3´		64
	Interrogation	5´- aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaKTACCAGC AGGTTGCCTCCC-3´	65	64
P6	Forward	5´-CTAGCTCAGTTCTTTCTCTGC-3´	265	64
	Reverse	5´-CCCAACTCCCAACCCGATC-3´		64
	Interrogation	5´- aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaTTACCAGCA GGTTGCCTCCC-3´	70	64
P7	Forward	5´-TCAGTAGGAAGCGGGTCGGG-3´	291	64
	Reverse	5´-GGCTGGGGAAGGGCAGAGTT-3´		64
	Interrogation	5´- aaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaaA TTACCAGCAGGTTGCCTCCC-3´	75	64

The PCR fragments were amplified from 20 ng of genomic DNA in a total volume of 20 µL with 10 µM of each dNTP, 2 mM MgCl2, PCR primers in various concentrations (10-25 fM/µL), and 0.5 U µL HotStartTaq DNA polymerase (Qiagen, Inc.). The PCR cycling profile was carried out as follows: initial denaturation at 95 ºC for 10 min followed by 35 cycles of denaturation at 94 ºC for 30 s, primer annealing at 64 ºC for 30 s, and elongation at 72 ºC for 1 min. Final extension was at 72 ºC for 3 min. To remove any remaining single-stranded primers and dNTPs, 2 µL of the PCR products was treated with 5 µL Antarctic phosphatase-exonuclease I buffer at 37 ºC for 15 min, then incubated with 7 µL Antarctic phosphatase buffer at 37 ºC for 15 min, and finally heat-treated at 80 ºC for 20 min. The PCR products were electrophoresed on agarose gels to verify specificity and to rule out any artefact bands.

Single Nucleotide Polymorphism (SNP) Reaction

Allele Separation and SNP Genotyping

Alleles were separated and detected using the GenomeLab™ SNPStart Primer Extension Kit and SNP locus tags via capillary electrophoresis on the Beckman Coulter CEQ™ 8000 Series Genetic Analysis System. The software *SNP Analysis Parameters*(https://www.beckmancoulter.com/wsrportal/bibliography?docname=A-10484A.pdf) was employed to analyse the data generated. Apparent fragment size and allele ID options were added and the samples on an ABI 3100 genetic analyzer (Applied Biosystems). Further processing by Genemapper software (Applied Biosystems) and multiple sequence alignments were carried out using DNAMAN (version 6.0, Lynnon Biosoft, USA) to confirm allele separation and SNP genotyping. Genotype frequencies were tested for deviations from the Hardy-Weinberg equilibrium using χ^2 tests.

Statistical Analysis

The frequency of each SNP was coded as present (1) or absent (0) for each animal's genotype, and threshold non-linear model procedures were performed in SAS (SAS Inst., NC) to analyse the data. Then a generalised linear model (PROC GLM, SAS Inst., NC) was used to fit the fixed effects of SNP, sire-breed, sex, supplementation level, and their interactions on SNP frequency distributions, growth, and body conformation parameters. Significance levels were tested at $P<0.01$ and $P<0.05$ using Tukey pairwise comparison thresholds.

Results

The PCR and interrogation primers designed for amplifying the ovine *ADRB3* gene (Table 7.1) worked well on all blood samples under the optimised platform.

Table 7.2. Least square means and standard errors (LSM±SE) of chest girth, withers height, body length, body condition score, live-weight, and average daily gain in *Spirulina* supplemented lambs

	***Spirulina* treatment group**						***p*-values**
	Control- 0 (n=16)		Medium-10% (n=16)		High-20% (n=16)		*Spirulina*
CG (cm)	95.0	± 0.6	95.6	± 0.6	96.1	± 0.7	0.376^{ns}
WH (cm)	62.9	± 0.4	62.7	± 0.4	63.1	± 0.3	0.669^{ns}
BL (cm)	65.7^{b}	± 0.4	66.6^{a}	± 0.4	66.8^{a}	± 0.4	0.015^{*}
BCS (0-5)	3.2^{b}	± 0.1	3.3^{b}	± 0.0	3.4^{a}	± 0.1	0.001^{***}
LWT (kg)	40.6^{b}	± 0.7	41.9^{a}	± 0.7	40.8^{b}	± 0.6	0.018^{*}
ADG (kg/d)	0.1	± 0.0	0.2	± 0.0	0.1	± 0.0	0.759^{ns}

Column means within a fixed effect bearing different superscripts significantly differ ($P<0.05$). Chest girth (CG), withers height (WH), body length (BL), body condition score (BCS), liveweight (LWT), and average daily weight gain (ADG). Level of significance: ns not significant ($P>0.05$), * significant ($P<0.05$), ** highly significant ($P<0.01$), and *** very highly significant ($P<0.001$).

Spirulina Supplementation and Phenotypic Data

As depicted in Table 7.2, *Spirulina* supplementation enabled sheep to have greater BL than the control group ($P<0.015$). Furthermore, lambs in the high (200mL/head/day of 20%wt/vol) *Spirulina* supplementation treatment group had greater BCS than the low (100mL/head/day of 10%wt/vol) and 0% (control) treatment groups ($P<0.001$). It was observed that sheep receiving low *Spirulina* supplementation had the heaviest LWT, 41.9 kg ($P< .018$); however, no difference was found between the high and control groups. The phenotypic results are shown in Table 7.2.

Allele and Genotype Frequencies

Three unique patterns were detected using 7 primer sets. All loci were heterozygous genotypes. The frequencies for alleles A, B, and C were 6.1%, 85.35%, and 8.49%, respectively. Allele B was present in all breeds, whereas allele A was found in White Suffolk, Dorset, and Merinos, and allele C was present in White Suffolk and Merinos only.

SNP Detection and Mutation Types

Fragment analysis and sequence comparison revealed that variations in the ovine ADRB3 gene occurred in both the coding and non-coding regions. Single Nucleotide polymorphism locations in the ADRB3 gene and mutation types are listed in Table 7.3.

Table 7.3. Mutation types and SNP locations in exon 1 and intron of the ovine *ADRB3* gene in lambs.

SNPs in exon			
SNP	Location	Type	Amino acid change
SNP 1	25A Del	Ins/Del	-
SNP 2	119 C>G	Transversion	Ala40Gly
SNP 3	130 C>T	Transition	None
SNP 4	156 G>A	Transition	None
SNP 5	379 T>A	Transversion	Ser127Thr
SNP 6	1024 T>A	Transversion	Tyr342Asn
SNP 7	1036 A>T	Transversion	Asn346Tyr
SNP 8	1072 C>G	Transversion	Arg358Gly
SNP 9	1082 T>G	Transversion	Phe361Cys
SNPs in intron			
SNP 10	1617 T>C	Transition	-
SNP 11	1733 A>G>C	Transversion or Transition	-

In total, 11 SNPs were identified, of which 9 were present in the exon, and the other two were in the intron. From these eleven SNPs, one indel, two transitions, six transversions in exon I, and one transition in the intron were found. In addition, the remaining SNP belonged to the three-base substitution class (1733 A>G>C), which was found in the intron that could have been either a transition or transversion. The six transversions in exon I changed the amino acid composition.

Table 7.4 SNP frequencies and numbers identified by lamb breed and sex.

SNPs	Breed				Sex		*p*-values	
	BS (n=12)	WS (n=12)	D (n=12)	M (n=12)	W (24)	E (24)	Breed	Sex
SNP 1	0^{b}	0.17^{a}	0.17^{a}	0.17^{a}	0.17^{a}	0.08^{b}	0.0001^{***}	0.0015^{**}
SNP 2	0.17^{d}	0.5^{c}	0.67^{b}	0.83^{a}	0.5^{b}	0.58^{a}	0.0001^{***}	0.0291^{*}
SNP 3	0^{b}	0^{b}	0^{b}	0.17^{a}	0.08^{a}	0^{b}	0.0001^{***}	0.0001^{***}
SNP 4	0^{b}	0.17^{a}	0^{b}	0.17^{a}	0.08^{a}	0.08^{a}	0.0001^{***}	1.000^{ns}
SNP 5	0.67^{b}	0.67^{b}	0.83^{c}	0.33^{a}	0.67^{a}	0.58^{b}	0.0001^{***}	0.0194^{*}
SNP 6	0^{b}	0^{b}	0^{b}	0.17^{a}	0.08^{a}	0^{b}	0.0001^{***}	0.0001^{***}
SNP 7	0.17^{c}	0.33^{b}	0.83^{a}	0.83^{a}	0.5^{b}	0.58^{a}	0.0001^{***}	0.0017^{**}
SNP 8	0.5^{b}	0.83^{a}	0.83^{a}	0.83^{a}	0.75^{a}	0.75^{a}	0.0001^{***}	1.000^{ns}
SNP 9	0^{b}	0^{b}	0^{b}	0.67^{a}	0.25^{a}	0.08^{b}	0.0001^{***}	0.0001^{***}
SNP 10	0^{b}	0.17^{a}	0^{b}	0^{b}	0^{b}	0.08^{a}	0.0001^{***}	0.0001^{***}
SNP 11	0.17^{c}	0.17^{c}	0.83^{a}	0.67^{b}	0.5^{a}	0.42^{b}	0.0001^{***}	0.0291^{*}
∑ SNP	1.67^{d}	3^{c}	4.17^{b}	4.83^{a}	3.58^{a}	3.25^{b}	0.0001^{***}	0.0081^{**}

BS=Black Suffolk, WS=White Suffolk, D=Dorset, M=Merino

SNP Number Detection

The average SNP numbers in different breeds and sexes and their associated p-values are shown in Table 7.4. Differences existed between breeds and sexes, and the GLM test showed that the differences were highly significant in breeds (P<0.0001) and sexes (P<0.0081). Purebred Merino sheep had the maximum average SNP number (4.83), and Black Suffolk crossbreds had the minimum (1.67). The average SNP numbers in White Suffolk and Dorset crossbreds were 3.0 and 4.17, respectively. Tukey's pairwise mean comparison test indicated that these significant breed differences resulted mainly from all breeds (Table 7.4). In addition, the number of SNPs was higher in wethers (3.58) than ewes (3.25).

SNP Frequencies

The frequency of SNPs in the various breeds and sexes (exon I) are shown in Figures 8.1 and 8.2, respectively. SNP1 (25 A Del) occurred with significantly higher (P<0.0001) frequency in White Suffolk (WS), Dorset (D) and Merino (M) breeds than Black Suffolk (BS). In addition, SNP1 was expressed with significantly higher frequency (P<0.0015) in wethers (0.15) than in ewes (0.08). The 119 C>G (SNP 2) presented in all breeds as a missense mutation which caused an Ala40Gly mutation. The maximum frequency of SNP 2 was found to be significantly higher (P<0.0001) in M (0.83) than in D (0.67), WS (0.50), or BS (0.17). It had a slightly higher frequency in ewes (0.58) than in wethers (0.50).

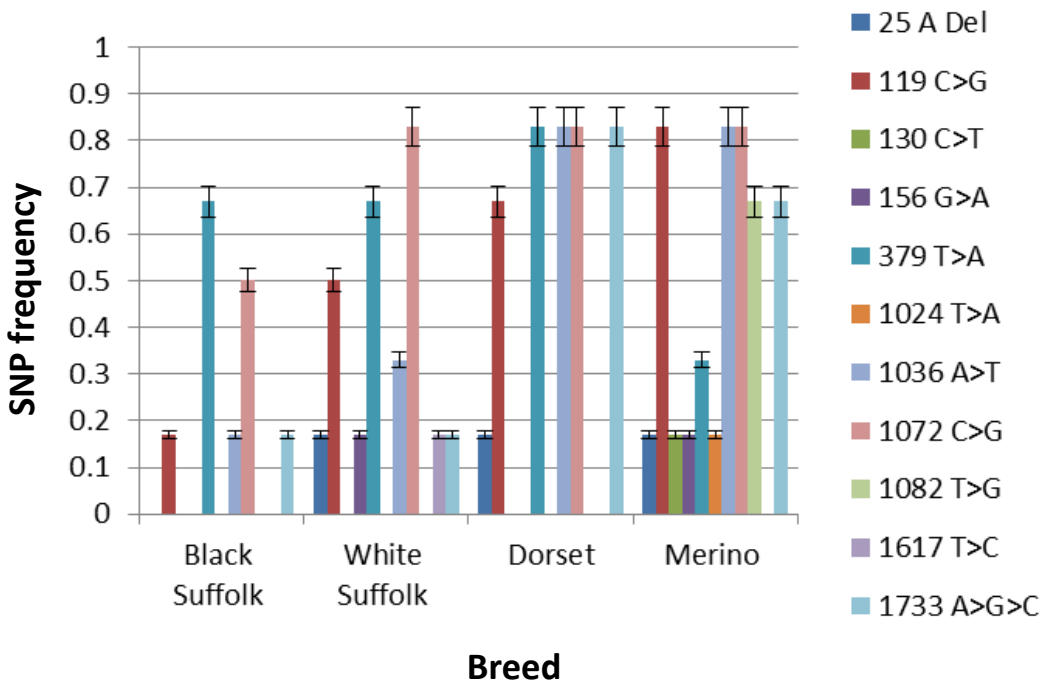

Figure 7.1 SNP frequencies in exon 1 and intron 1 by breed of sheep

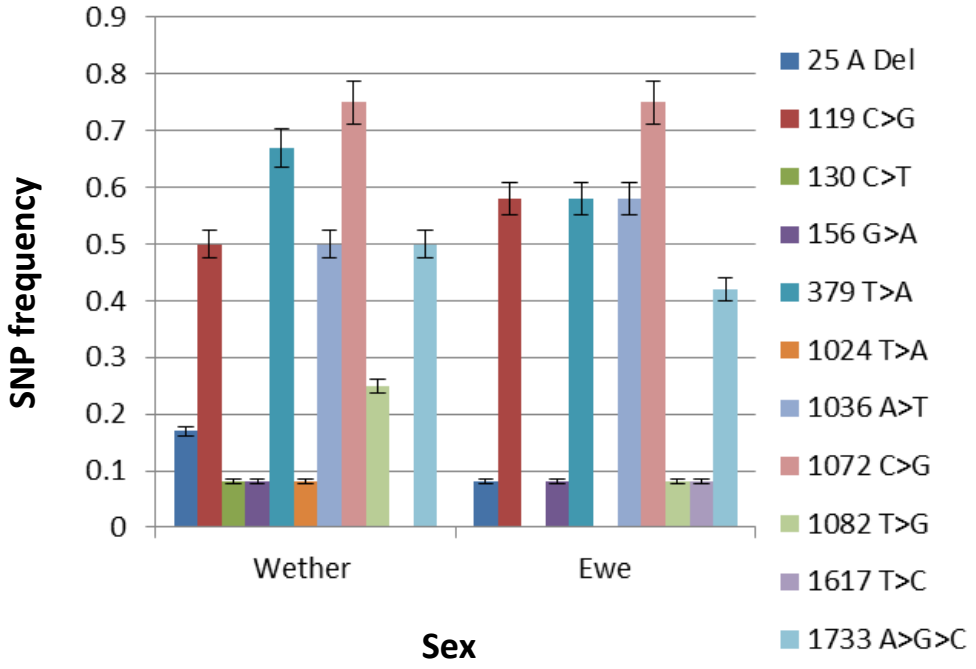

Figure 7.2 SNP frequencies in exon 1 and intron in wethers and ewes

The frequency of SNP3 was greatest among M sheep (0.17) and this breed difference was highly significant (P<0.0001). The 130 C>T (SNP3) was a transition type mutation that did not occur in ewes, although it had a significant frequency (P<0.0001) in wethers (0.08). Similarly, SNP4 (156 G>A) was a transition type mutation, but was found in both M (0.17) and WS (0.17) breeds with significant breed differences in frequencies (P< 0.0001). It presented equally in both wethers (0.80) and ewes (0.80); thus, no significant difference was observed. A missense mutation, SNP5 (379 T>A), resulted in a Ser127Thr mutation. It significantly (P<0.0001) differed in all breeds (D, 0.83; both BS and WS. 0.67; and M, 0.33) and was significantly higher (P<0.0194) in wethers (0.67) than ewes (0.58).

SNP6 (1024 T>A) was found exclusively in M sheep (0.17) and significantly differed (P<0.0001) from other breeds. It was a transversion mutation that caused a missense mutation of Tyr342Asn. Its frequency was observed only in wethers (0.08) and was significantly

different (P<0.0001) from ewes. The 1036 A>T (SNP7) was discovered in all breeds with significantly higher (P<0.0001) frequencies in both D and M (0.83), WS (0.33), and BS (0.17). Its frequency was observed to be significantly (P<0.0017) higher in ewes (0.58) than wethers (0.50).

SNP8 was detected in all breeds, but occurred at a high frequency in WS, D, and M (0.83 each) and a lower frequency in BS (0.50). The 1072 C>G (SNP8) mutation was a missense Arg358Gly mutation. Its frequency significantly differed (P<0.0001) in all breeds. There was no significant sex difference between lambs. Another missense mutation, 1082 T>G (SNP9), that resulted in Phe361Cys, was detected exclusively in the M (0.67) breed. Its frequency significantly differed (P<0.0001) between breeds and sexes and was higher in wethers (0.25) than ewes (0.08).

In the intron, SNP10 (1617 T>C) was a transition mutation that mainly presented in WS (0.17) and significantly differed from other breeds (P<0.0001). It was detected only in ewes (0.08) and significantly differed from wethers (P<0.0001). Finally, the 1733 A>G>C (SNP11) is a tri-base mutation seen in all breeds, but with significantly higher (P<0.0001) frequency in D (0.83), M (0.67), WS (0.17), and BS (0.17). Its frequency was significantly (P<0.0291) higher in wethers (0.50) than ewes (0.42).

Discussion

This experiment is a follow-up to our previous results that demonstrated that the live-weight and body conformation measurements of Australian crossbred sheep can be boosted by using protein-rich *Spirulina* as a dietary supplement (Holman *et al.*, 2012). In agreement with the

previous data, Table 7.4 demonstrated that at low level of *Spirulina* supplementation, there was an increase in liveweight and body conformation traits (Table 7.2). However, excessive *Spirulina* supplementation can suppress optimal sheep growth because the excess protein is deaminated and gets lost in the urine or broken down in the liver, leading to fatty liver and ketosis (Holman *et al.*, 2014). In addition, we confirmed the importance of sire breed selection in breeding programmes because of variations in genetic predisposition for muscle growth as opposed to body fat deposition (Holman *et al.*, 2014b).

The current study describes polymorphisms (i.e., SNP variation) in both coding and non-coding regions of the ovine *ADRB3* gene in purebred and crossbred Australian sheep and its association with *Spirulina* supplementation. Indel mutations play key roles in the ovine genome and can result in frameshift mutations of the amino acid sequence. However, a comprehensive understanding of how the indels influence the phenotype and impact evolutionary processes is not clearly understood (Wu *et al.*, 2012). SNP2 (119 C>G) mutation resulted in Ala40Gly substitution, which is a change from the hydrophobic alanine (A) to glycine (G) at position 40. The SNP5 (379T>A) mutation is responsible for Ser127Thr substitution. This substitution is a change from the small sized and polar Serine (S) to the medium sized and polar Threonine (T) at position 127. These two substitutions are located in the first and third transmembrane segments in the primary structure of the ovine *ADRB3* locus, respectively, and they have residues common to all two β-adrenergic receptor subtypes (Strosberg, 1997; Wu *et al.*, 2012). These amino acid substitutions are thought to be involved in ligand binding (Strosberg, 1997; Wu *et al.*, 2012). The finding that SNP2 was distributed at a higher frequency in purebred Merino (M) than in other breeds, and that SNP5 distribution was higher in Dorset (D) crossbreds, is consistent with a recently published study that reported higher frequencies of these substitutions in dual-purpose meat and wool sheep breeds (Wu *et al.*, 2012).

Both SNP6 and SNP7 are responsible for Tyr342Asn and Asn346Tyr substitutions, respectively, and are located in the seventh transmembrane segment of the ovine *ADRB*3 gene (Strosberg, 1997; Waldhoer *et al.*, 2003). It has been shown that some residues in this segment are vital for receptor activation by *ADRB*3 agonists (Granneman *et al.*, 1998) and for ligand binding (Strosberg, 1997). Both substitutions, Arg358Gly (SNP8) and Phe361Cys (SNP9), are located in the carboxyl terminal region of the *ADRB*3 receptor. This region plays a crucial role in cell signalling efficiency (Waldhoer *et al.*, 2003) and houses the receptor sites for coupling with the G-protein (Strosberg, 1997). These Arg358Gly and Phe361Cys substitutions can result in the change or loss of function of the *ADRB*3 receptor. It has been demonstrated that the oral administration of β-adrenergic agonists increased muscle accretion and decreased fat deposition in pigs and cattle (Mersmann, 1998); thus, the functional change can affect meat production and quality. The mutation frequency of SNP8 was low in Black Suffolk (BS) crossbreds and high in White Suffolk (WS) and D crossbreds as well as purebred M lambs. The Phe361Cys (SNP9) frequency was detected only in M lambs. In the intron, SNP10 was detected in WS crossbreds only, while SNP3 was only detected in M purebreds, suggesting the use of these SNPs as molecular markers for breeding programs. The total SNP frequencies significantly differed between lamb breeds; therefore, they reinforced the underlying fact that polymorphisms in the *ADRB*3 gene can affect the expression, tissue distribution, thermogenic, and lipolytic capabilities of the receptor (Forrest *et al.*, 2007; 2009; Forrest and Hickford, 2000).

There is a significant association between the Trp64Arg mutation in the human *ADRB*3 gene with obesity (Brettfeld *et al.*, 2012; Clément *et al.*, 1995; Takenaka *et al.*, 2012), insulin resistance and hyperuricemia (Malik *et al.*, 2011; Morcillo *et al.*, 2010). The associations

between ovine *ADRB*3 polymorphism with birth weight, growth rate, carcass composition, lamb mortality and cold survival, and wool mean staple strength and yield have been confirmed (Forrest *et al.*, 2003; 2007; 2009). This current study established the existence of a significant SNP difference between breeds of Australian purebred and crossbred sheep and their importance in changing the *ADRB*3 gene function. In this study, we detected less mutations in the BS breed compared to other breeds, particularly D crossbreds and M purebreds. These mutations result in amino acid changes that may increase fat deposition in sheep tissues. Thus, in conjunction with our previous findings from the *Spirulina* supplementation experiment, we suggest the use of a combination of BS breed and low *Spirulina* supplementation in order to increase lamb production with less fat content in the meat. However, further fatty acid profiling, mRNA expression, and proteomic analyses are required to fully understand the underlying mechanisms of *ADRB*3 SNPs and gene expression in response to *Spirulina* supplementation.

Conclusion

The ovine *ADRB*3 gene was investigated, several SNPs were identified, and their association with *Spirulina* supplementation level was evaluated in purebred and crossbred lambs. The identified SNPs in both coding and non-coding regions and their distributions in four sheep breeds reflected existing genetic variation. In conclusion, the genetics-nutrition combination of supplementing Black Suffolk crossbred lambs with 100mL/head/day of 10%wt/vol of *Spirulina* may be used to increase lean meat production in the Australian prime lamb industry. Therefore, the hypothesis that genetics-nutrition interactions between sheep breeds with fewer mutations at the ADRB3 locus and an optimal Spirulina supplementation level will increase lean meat production holds true and should be accepted.

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Chapter 8

General Discussion

The present study addressed the biological aspects of depot-specific fat accretion and metabolism in sheep based on an experimental trial with four genetically diverse sheep breeds and three levels of protein-rich dietary supplementation with Spirulina (*Arthrospira platensis*). The component of the study on the evaluation of mRNA expression levels of the *AANAT, ADRB3, BTG2* and *FASN* genes provided indisputable evidence for the significant effects of different levels of *Spirulina* supplementation on gene expression profiles in the liver, heart, kidney, muscle and adipose tissues and organs. The data also indicated that genetic background, and to a lesser extent, gender, determined the mRNA expression levels of *AANAT*, *ADRB3*, *BTG2* and *FASN* genes. The feeding trial phase of the experiment had earlier demonstrated that 10% level of supplementation with *Spirulina* resulted in a higher PUFA composition in the tested organs.

AANAT transcripts were found to be differentially expressed in high vs. low n-3 index (O3I) muscles, suggesting a role for melatonin in reducing oxidative damage, including that to PUFA (Holman et al., 2014; Perez et al., 2010). The ability of melatonin to protect against lipid peroxidation has been documented in many studies using animal and plant tissues (Holman *et al.*, 2014). Recently, Spanish scientists revealed that melatonin consumption assists in the control of weight gain since it stimulates the appearance of brown fat, a type of fat cell that burns calories instead of storing them (Jiménez-Aranda *et al.*, 2013). Their research demonstrated that melatonin treatment not only induced browning of inguinal white adipose tissue in Zucker diabetic fatty rats, but also increased the thermogenic activity of this tissue

(Jiménez-Aranda *et al.*, 2013). Taken together, these findings highlight the anti-obesity effect of melatonin and explain its metabolic benefits of protection against oxidative degradation of PUFA in muscle tissue, which produce higher O3I levels (Jiménez-Aranda *et al.*, 2013). *Spirulina* supplementation is effective in changing the levels of *AANAT* transcription. In this study, 20% *Spirulina* supplementation resulted in a 7-fold and 10% *Spirulina* treatments resulted in 20-fold up-regulation of *AANAT* mRNA levels compared to the control group. Therefore, feeding sheep with 20% Spirulina supplementation may result in weight loss due to the enhanced production of melatonin in their muscle tissue. High level of *Spirulina* supplementation (20%) compared to the 0% and 10% groups were significantly up-regulating the *AANAT* expression levels in the kidney. Expression of the *AANAT* encoded protein accelerates the production of melatonin hormone by catalysing the rate-limiting step in the synthesis of melatonin from serotonin (Coon et al., 1999, Reiter et al., 2014). The anti-obesity effect of melatonin and its protective role against oxidative degradation of PUFA (Jiménez-Aranda et al., 2013, Reiter et al., 2014) agree with the observations in the present study as both n-3 and n-6 PUFA levels increased and ∑SFA decreased in the kidney of lambs supplemented with 10% and 20% dietary *Spirulina*. The medium level (10%) of *Spirulina* supplementation also proved to be more beneficial for higher productive performance by fattening sheep rather than the high (20%) *Spirulina* supplementation. This is due to the significant up-regulation of *ADRB3* which has been shown to be linked with obesity through regulation of lipid metabolism in humans and other mammals. Supplementing sheep with a higher dosage of *Spirulina* may result in lower efficiency in liver function due to the breakdown of extra protein resulting to fatty liver and ketosis. It could also lead to a decrease in total production because of the negative correlation between protein accretion and fat deposition rates as a result of the over expression of *BTG2* by the high level of *Spirulina*. This gene has a potential role in muscle fibre size, intramuscular fat deposition and weight loss (Kamaid and Giráldez, 2008, Mo et al., 2011).

ADRB3 transcription levels were significantly up-regulated in the kidney of prime lambs supplemented with 20% dietary *Spirulina*, which is consistent with the observed FA results. This suggests that both the 10% and 20% levels of dietary *Spirulina* supplementation increased n-3 and n-6 PUFA compositions and decreased ∑SFA content in the kidney of lambs. However, in the liver, *ADRB3* transcripts were found to be differentially expressed in the 10% compared to the 0% and 20% *Spirulina* groups. Given that the *ADRB3* gene encodes proteins regulating mammalian energy storage and expenditure by mediating effects from the sympathetic nervous system (Hu et al., 2010, Wu et al., 2012), the results suggest an intricate genetics-nutrition interaction underpinning transcription at the molecular level that can be manipulated through diet in order to achieve healthy FA composition outcomes.

FA analyses demonstrated that 10% and 20% *Spirulina* levels increased n-3 and n-6 FAs in the liver of lambs due to the up-regulation of both *ADRB3* and *BTG2* transcriptions. This seems to suggest that dietary *Spirulina* supplementation increases metabolic rate and lipolysis in the liver through up-regulation of the *ADRB3* gene and simultaneously induces a decline in preadipocyte proliferation, an increase in energy expenditure and a decline in energy uptake in adipocytes, ultimately enhancing n-3 and n-6 PUFA contents in the liver. *Spirulina* supplementation both in medium and high levels resulted in an increase in body weight mainly due to an increases in transcription levels of *FASN* in both SAT and muscle tissues. This gene contributes to the regulation of body weight in humans and results in the development of obesity (Berndt et al., 2007, Boizard et al., 1998). Therefore, supplementing sheep with 10% *Spirulina* can increase lamb production as a result of heightened transcription levels of *ADRB3* and *FASN* genes in SAT. Also, this might beneficially alter the fatty acid profile by reducing the oxidation of PUFA in skeletal muscle.

The low level of *Spirulina* supplementation increased the transcription level of *FASN* gene in the heart which might have contributed to an increase in n-6 and n-9 PUFA by utilizing palmitic acid, which is the first FA produced during FA synthesis as a precursor for longer FAs. This indicates that low *Spirulina* supplementation may result in a decrease in saturated FA and a concomitant increase in PUFA in the heart of supplemented lambs.

The observed mRNA transcription of the *AANAT, ADRB3, BTG2* and *FASN* gene expression levels are also consistent with the observed phenotypic data indicating that the reductions in fat deposition, especially in subcutaneous adipose and muscle tissues, were related to the significant decrease of *FASN* and *ADRB3* mRNA expression levels in tissues receiving *Spirulina* supplementation. *ADRB3* receptor and *FASN* genes are responsible for fat synthesis; therefore, not only did dietary *Spirulina* not compensate the feed loss, but it also reduced their transcription levels leading to production losses. On the other hand, the increase of the *AANAT* transcriptions indicated a possible increase of melatonin production, which is responsible for weight loss, in both subcutaneous adipose and muscle tissues. The observed decreases in transcription levels of *BTG2* suggest extra energy expenditure and less fat deposition in lamb tissues receiving dietary *Spirulina* supplement. These findings indicate that the dietary *Spirulina* cannot solely compensate feed loss, but should be utilized with good quality feed to both increase lamb production and PUFA enhancement.

It was also evident that the medium-level *Spirulina* diet resulted in higher polyunsaturated fatty acid compositions; increased eicosapentaenoic acid and linoleic acid in subcutaneous adipose tissue, which increased the percentage of n-*6* PUFA in Australian dual-purpose lambs. Research has demonstrated that eicosapentaenoic acid is an essential and physiologically significant n-3 fatty acid (Abeywardena and Patten, 2011, Belay et al., 1993, Daley et al., 2010,

Mapiye et al., 2011, Woods and Fearon, 2009). It was discovered that the medium *Spirulina* diet increased eicosapentaenoic acid in subcutaneous adipose, muscle, and liver tissues. EPA is one of the types of n-3 needed by the human body, which cannot make EPA fatty acid and must thus acquire it from food or supplement sources. The low *Spirulina* diet reduced the percentage of pentadecanoic acid in heart tissue (Hoashi et al., 2008, Scollan et al., 2001). This is because the *Spirulina* supplement is rich in α-Linolenic acid. This is also demonstrated in this study that the low-level *Spirulina* diet reduces the conversion of unsaturated fatty acids to saturated fatty acids by the biohydrogenation process (Kouba and Mourot, 2011, Pethick et al., 2010) in crossbred lambs. An increase in eicosadienoic acid (20:2n-6) was observed in the tissues associated with the low-level *Spirulina* diet.

It was also demonstrated that the medium *Spirulina* diet significantly affected the percentage of docosapentaenoic acid in kidney tissue. Docosapentaenoic acid is an n-6 FA (Kouba and Mourot, 2011). It is formed metabolically from LA and is a constituent of animal glycerophospholipids (Scollan et al., 2001, Smet et al., 2004, Wachira et al., 2007). This highlights that a *Spirulina* diet rich in LA increases other n-6 PUFAs in Australian crossbred lambs, all the way through 22:5n-6. The *Spirulina* diets were shown to affect the composition of oleic acid in *longissimus dorsi* muscle tissue. Oleic acid is classified as a monounsaturated *n*-9 FA (Daley et al., 2010, Kouba and Mourot, 2011). Previous studies have found significant variation of FA composition in response to dietary supplementation (Alfaia et al., 2009, Doreau et al., 2010, Mapiye et al., 2011, Scollan et al., 2001). The biosynthesis of oleic acid involves the action of the enzyme stearoyl-CoA 9-desaturase (Wood *et al.*, 2008) acting on stearoyl-CoA (Price et al., 2000, Woods and Fearon, 2009) and the dehydrogenation of stearic acid to give the MUFA derivative oleic acid (Wood et al., 2008, Wood et al., 2004, Woods and Fearon, 2009).

The Spirulina diet resulted in the reduction of 16:0 fatty acids, which is known as palmitic acid. This acid is the first produced during fatty acid synthesis (Wood et al., 2008, Wood et al., 2004). Palmitic acid is a precursor to longer fatty acids (Wachira et al., 2002). It negatively feeds back on acetyl-CoA carboxylase (ACC) (Kouba and Mourot, 2011, Santos-Silva et al., 2002), which is responsible for converting acetyl-CoA to malonyl-CoA (Smet et al., 2004, Wood and Enser, 1997), which in turn is used to add to the growing acyl chain, thus preventing further palmitate generation (Price et al., 2000, Raes et al., 2004, Rowe, 2010). Research has demonstrated that palmitic acid increases the risk of developing cardiovascular diseases (Abeywardena and Patten, 2011, Belay et al., 1993, Fokkema et al., 2002). In this study, the tissue and composition of lamb muscle and heart were observed to respond to the medium Spirulina diet supplementation, which resulted in the lowest levels of palmitic acid and highest levels of linoleic acid and n-6 PUFA.

The component that investigated the impact of *Spirulina* supplementation under simulated drought condition demonstrated that the 10% level of supplementation caused sheep to lose weight and decreased the body measurements factors. This was mostly attributable to the lack of good quality pasture and fibre in the animals' diet. It was evident that excessively high dietary protein intake can restrain sheep growth due to the negative correlation between protein accretion and fat deposition rates (Holman *et al.*, 2012). The study also revealed the existence of a significant SNP difference between breeds of Australian purebred and crossbred sheep and their role in changing *ADRB*3 gene function. Furthermore, less mutations were detected in the BS compared to other breeds, particularly D crossbreds and M purebreds. These mutations result in amino acid changes that increase fat deposition in sheep tissues. Thus, in conjunction with previous findings from the *Spirulina* supplementation experiment, the use of a combination of BS breed and low *Spirulina* supplementation is suggested in order to increase lamb production with less fat content in the meat. However, further fatty acid profiling, mRNA expression, and proteomic analyses are required to fully

understand the underlying mechanisms of *ADRB*3 SNPs and gene expression in response to *Spirulina* supplementation.

Chapter 9

General conclusions and implications

This study identified variations in the FA profiles of lamb adipose, muscle, heart, kidney and liver tissues and organs that are attributable to differences in the level of *Spirulina* supplementation. The 10% level of *Spirulina* supplementation increased FA composition in some tissues and elevated the proportion of n-3 and n-6 PUFA in all tissues and organs. Low and high levels of *Spirulina* supplementation slightly increased PUFAs in some tissues and organs, but excessive supplementation can suppress optimal sheep growth because the excess protein is deaminated and lost in the urine or broken down in the liver, leading to fatty liver and ketosis. The use of 10% *Spirulina* supplementation in order to increase lamb production with higher PUFA composition is recommended.

Chapter 2 reviewed the applications of real-time RT-qPCR technique for the detection of mRNA and gene expression levels in various research fields. The extreme sensitivity of this technique was explained to be its weak point. RT-qPCR is a technique that thrives in gene expression investigations related to nutritional genomics and nutritional studies such as those examining n-3 and n-6 fatty acid syntheses. In Chapter 2, real-time RT-qPCR technique was used to identify expression profiles of genes that could affect fat and protein biosynthesis and metabolism. The investigated tissues were: liver, heart, muscle, kidney, and adipose tissues in genetically divergent purebred and crossbred Australian sheep that were supplemented with *Spirulina*.

The objectives of the study described in Chapter 3 were to examine the effect of adding *Spirulina* in the diet on the expression of AANAT, ADRB3, BTG2, and FASN genes in the subcutaneous adipose (SAT) and muscle (ld) tissues of sheep. It was evident that when *Spirulina* was added at levels of 10 and 20%, the transcription of all the selected genes in both SAT and ld tissues was strongly up-regulated. In the aforementioned tissues, sheep breed and sex were two factors that did not present considerable influence on the gene expression patterns. Sheep supplemented with 20% *Spirulina* showed a decrease in intramuscular fat content. This result is likely due to the production of more melatonin in sheep muscle tissues, which is stimulated by the expression of BTG2 in SAT. However, supplementation with 10% *Spirulina* increased lamb production, and was also accompanied by higher long-chain PUFA content in kidney, liver and heart.

The study in Chapter 4 was carried out to assess the molecular genetics and genomics-nutrition interactions in fatty acid composition variations and the effects of dietary supplementation of lambs with *Spirulina* (*Arthrospira platensis*) on the mRNA expression and transcriptional analysis of *AANAT, ADRB3, BTG2 and FASN* genes in the heart, kidney and liver. It was concluded that mRNA expression levels of *AANAT*, *ADRB3*, *BTG2*, and *FASN* genes in the heart, kidney and liver were mainly influenced by dietary *Spirulina* supplementation level. Taken together, the results showed that fatty acid metabolism in the kidney and liver are more sensitive to dietary manipulation than in the heart. These findings support the use of the 10% dietary *Spirulina* level of supplementation for optimal increase in the health-benefitting n-3 and n-6 fatty acid contents of organs in Australian purebred and crossbred sheep.

In Chapter 5, the effects of *Spirulina* supplementation level on the expression of fat metabolism genes under simulated drought conditions in purebred and crossbred Australian sheep was investigated. The objective was to evaluate the potential of dietary *Spirulina* as an alternative supplement during times of feed shortage. Phenotypic and body measurement data indicated that dietary *Spirulina* compensated for feed loss. However, at high levels of supplementation, it resulted in a reduction in meat production due to weight loss. The results of transcription levels of the *AANAT, ADRB3, BGT2,* and *FASN* genes confirmed the significant decline in fat deposition with *Spirulina* supplementation during simulated drought conditions. Dietary *Spirulina* can benefit sheep production and PUFA enhancement when good quality pasture is available.

In order to confirm the potential of n-3 and n-6 enhancement, Chapter 6 investigated the fatty acid profiles of sheep tissues and organs under different levels of *Spirulina* supplementation. The results demonstrated that 10% dietary supplementation with *Spirulina* significantly increased the n-3 and n-6 profiles of PUFA, mainly in the subcutaneous adipose and muscle tissues. These findings strengthened the demonstration of the transcription levels of *AANAT, ADRB3, BTG2,* and *FASN* genes.

Finally, polymorphism of the *ADRB3* gene was demonstrated to be responsible for obesity and fatness. Black Suffolk sheep had a lower *ADRB3* mutation rate compared to White Suffolk, Dorset, and Merino sheep. The results from body conformation traits demonstrated that Black Suffolk sheep responded to 10% dietary *Spirulina* and increased lamb production.

Areas to be researched in the future

The research undertaken in this thesis has demonstrated the potential utility and benefits of *Spirulina* supplementation with Australian lamb. Additional research and development is still required in:

- Defining the optimal balance of n-3 PUFA and antioxidants in ruminant products
- Research on how animal factors (age, sex, breed) affect the response to the test ingredient
- considering different diet formulations and feed management such as grazing in comparison with feedlotting

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